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A fibrin based hemostatic agent and wound dressing system specifically to be usable on the battlefield has been developed. The agent is a fibrin monomer based powder which is directly usable without any pre-mixing. In vivo tests indicate that: (1) the new hemostatic agent controls bleeding significantly faster than the only commercially available fibrin-based hemostatic agent, (Tisseel®, Immuno AG, Austria) when tested in a splenic injury model; (2) the tissue response and wound healing obtained with the new material and Tisseel® appear to be comparable; (3) the new material shows promise in the control of arterial bleeding when used on the surface of a hydrophilic sponge; and (4) the material yields a tissue tensile strength equivalent to surgical staples and with less localized inflammation at one week when it is used in an external, adhesive-backed wound dressing.

Because of its ease of use and effectiveness, this material has the potential for wide applicability in the treatment of trauma in civilian settings as well as its military uses.

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R.L.W

Robert L. Whalen, Ph.D.
April 18, 1997

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Introduction

The goal of this program is the development and testing of a fibrin-based tissue adhesive/hemostatic agent for controlling hemorrhage which will be usable on the battlefield. To be employable under field conditions, it is our view that the material must be usable in a dry form and not require pre-mixing of ingredients. Furthermore, it should also include delivery systems which protect and seal the site of injury from potential contamination. With the completion of animal testing in the second year of this program, we believe the program goals have been met.

In vivo testing of the new hemostatic agent was performed in the Department of Bioengineering at the Cleveland Clinic Foundation (CCF), Cleveland, OH. The animal test results indicate that: (1) the new hemostatic agent controls bleeding significantly faster than the only commercially available fibrin-based hemostatic agent, (Tisseel®, Immuno AG, Austria) when tested in a splenic injury model; (2) the tissue response and wound healing obtained with the new material and Tisseel® appear to be comparable; (3) the new material shows promise in the control of arterial bleeding when used on the surface of a hydrophilic sponge; and (4) the material yields a tissue tensile strength equivalent to surgical staples at one week and with less inflammation when it is used as part of an external, adhesive-backed wound dressing. A summary report from the CCF describing the test methodologies and presenting detailed results from this testing is included as Appendix A.

The delivery systems which were developed for the hemostatic agent are designed to minimize the amount of material actually used and to place the material in contact with the tissue. The external wound dressing also includes a timed release antimicrobial (chlorhexidine gluconate) to help reduce the incidence of infection. Bactericidal testing has shown this system produces significant reduction in bacterial counts.

Toxicity testing of delivery systems for the hemostatic agent was accomplished in the first year of this program. Those tests showed that the delivery systems meet USP Class VI standards. Similar toxicity testing of the hemostatic agent itself was recently completed. The hemostatic agent itself yielded negative results in ISO standardized tests of pyrogenicity, primary skin irritation, and systemic injection. It did induce activation of C3 and C5 human complement proteins, a likely consequence of the animal origin of the proteins used in its manufacture (bovine thrombin and fibrinogen). The results of the toxicity testing of the agent are included as Appendix B.

The new hemostatic agent/tissue adhesive and its method of manufacture have been successfully patented. U.S. Patent No. 5,464,471, which covers the formulation, its preparation, and the concept of both delivery systems for the material which were developed, was issued on November 15, 1995. A copy of that patent is included in Appendix C. Because of its utility in the treatment of trauma, we believe that the potential civilian uses for this product are highly significant. We are thus optimistic that this new material for controlling hemorrhage can be successfully commercialized in Phase III.

Overview

The basis for our approach to develop the new material was relatively straight forward. Figure 1 illustrates the normal course of events leading to the formation of a stable fibrin clot.

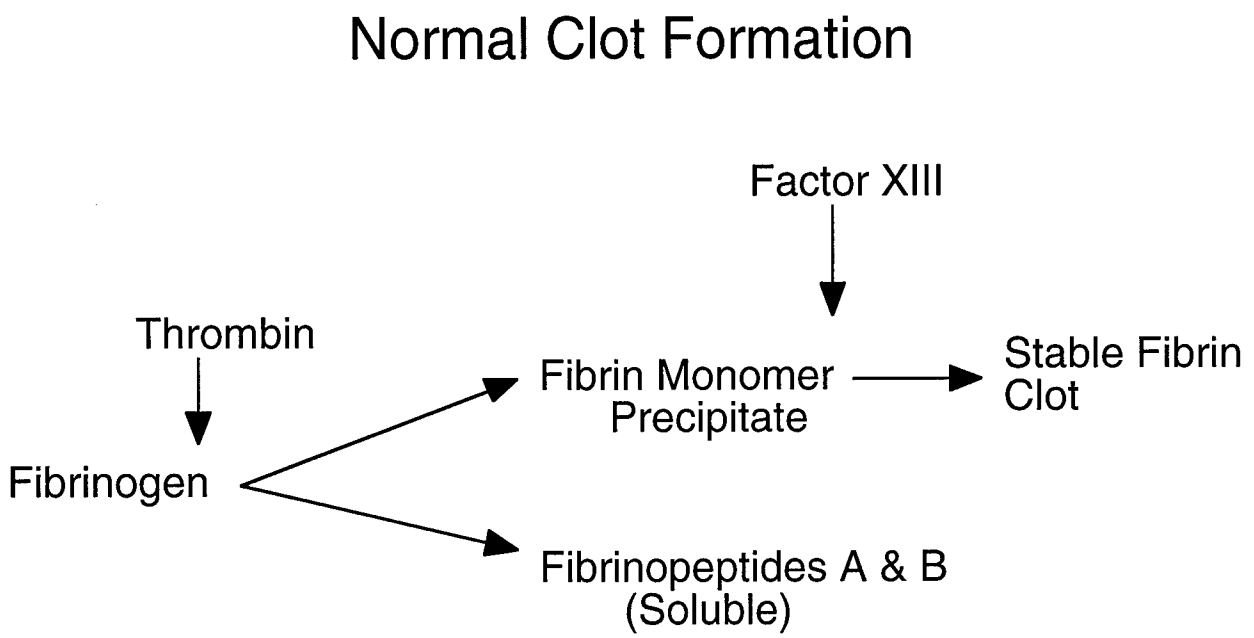

Figure 1. The normal course of events in the formation of a stable fibrin clot. The action of thrombin is also to activate Factor XIII.

Fibrinogen, the soluble precursor of fibrin, is a symmetrical molecule containing two a, two b, and two g chains. The chains are held together by disulfide bridges. The action of thrombin on fibrinogen is specific and limited. It cleaves an arginyl-glycine bond in each of the α and β chains releasing two A peptides and two B peptides. The remaining portion of the molecule is termed fibrin monomer. Fibrin monomer itself is soluble, but it spontaneously polymerizes as a result of the formation of hydrogen and hydrophobic bonds to form a precipitate. The bonds linking these fibrin monomer subunits are relatively weak, however, and may easily be disrupted by extremes of pH or the action of urea. Thrombin also activates Factor XIII, and it is the activated state of Factor XIII which forms covalent bonds between the fibrin monomers to make a stable fibrin clot.

In our process, we employ purified proteins to interrupt this process at the stage of the formation of the loosely bound fibrin monomer precipitate. In the absence of Factor XIII, we disrupt the weak hydrogen bonds which have produced the polymerized fibrin monomer to yield a solution of fibrin monomer itself, the essential subunit in clot formation. To this

solution we then add all other constituents of the new hemostatic agent so that we then have a single solution containing the necessary components.

When this solution is lyophilized, the result is an extremely low density powder (0.013 g/cm3). This powder is a homogeneous mixture of all constituents. In that form it may be: (1) applied directly to the site of bleeding to produce hemostasis, for controlling diffuse bleeding; (2) used with the hydrophilic sponge material which both absorbs water and provides mechanical compression to assist in obtaining hemostasis, for controlling arterial bleeding; or (3) used on the surface of an external wound dressing to stop bleeding and bond the tissue as an alternative to sutures.

While our primary focus in this program has been on the military applications of the new hemostatic agent/tissue adhesive system, it is obvious that the civilian uses for this type of product are significant. There is a definite need for improved hemostatic agents during emergency first aid in pre-hospital settings or even in emergency rooms.The numbers are impressive.

Physical trauma from accidents and violence is a leading cause of morbidity and mortality in the United States. Each year 57 million Americans are injured, resulting in visits to emergency rooms and trauma centers throughout the country. Many of these visits require some form of intervention for hemostasis.

Emergency rooms across the United States, not to mention the European Union, would find this product useful in hemostasis and tissue adhesion. Incorporating the agent into a sponge provides a form of the product particularly useful for the control of deep arterial bleeding, such as gunshot and penetrating wounds. Depending upon the region of the country, gunshot wounds constitute up to 26% of trauma cases in some hospitals. Decreasing morbidity and mortality from these wounds is clearly an important goal, and achieving early hemostasis is probably the single most important factor in improving survival rates. The new material developed in this program is a substance which is likely to prove extremely useful.

We believe that regulatory issues preventing the approval of fibrin tissue adhesives in this country will soon be fully addressed. In this SBIR program, we have employed animal protein (bovine) which will not result in disease transmission, but can have the potential for evoking an immune response. Recombinant human fibrinogen is to become commercially available, however, in the near future, and recombinant thrombin is already clinically available. It will then be possible to formulate the material utilizing no components derived from pooled human donor blood. The issues of disease transmission via the product or immune responses are thus circumvented, and regulatory approval of this product should be facilitated.

The new hemostatic agent and trauma-specific delivery systems we have developed would appear to represent readily commercialized products for use by ambulance personnel in pre-hospital settings and by physicians in emergency rooms. The clinical trials necessary to obtain FDA approval to market this product in the United States are beyond the scope of

this SBIR effort, but we believe the SBIR achievements have been be sufficient to justify continued investment in the product from private sources to continue its development in Phase III.

Task I. Formulation Development

In this task our objectives were to develop the most suitable formulation for the tissue adhesive, characterize analytically the complete formulation, and establish manufacturing and sterilization techniques which would result in consistent production. Utilizing gel permeation chromatography, we characterized the most suitable formulation, developed a reliable process for obtaining fibrin monomer to use in the tissue adhesive, and established appropriate methods of sterilization.

Process Development and Sterilization of the Fibrin Monomer

In order to develop and quantify a process for the development and sterilization of the fibrin monomer used in the tissue adhesive, it became necessary to identify and quantify the constituents in the preparation solution to ensure accurate production of the agent. Techniques to detect such plasma products have evolved with the development of highly sensitive degradation methods for protein sequence analysis. These microscale sequencing techniques demanded for equally sensitive techniques for purification, isolation, fragmentation and identification. For all of these purposes, high performance liquid chromatography (HPLC) proved to be an excellent tool.

A process for preparing fibrin monomer was defined and reported in previous reports. The hemostatic agent consists primarily of a lyophilized solution of fibrin monomer, aprotinin, thrombin, and calcium chloride.

We have examined the effects of adding aprotinin to the formulation and have determined that its addition to the formulation is of no benefit. Bovine aprotinin (Trasylol,® Bayer Corporation, West Haven, CT) was obtained in a 10,000 KIU/ml concentration. When added to the hemostatic agent, 5,000 KIU are used. For comparison, when aprotinin is used during cardiopulmonary bypass surgery, several million KIU are generally employed. Thus, 5,000 KIU represents a very low exposure to aprotinin.

To study its effect on acute hemostasis, we first conducted in vitro measurements using a modified activated clotting time test similar to the measurements made in Phase I. The results indicated that the addition of aprotinin to the fibrin formulation made no measurable difference.

As will be discussed in a following section, the addition of aprotinin to the hemostatic agent produced a measurable but statistically insignificant increase in the bleeding time measured in vivo. It also had no observable effect on healing when we examined the tissue histologically. On the basis of these results, there appears to be no basis for its addition to

the formulation. It is, however, a constituent in Tisseel.®

HPLC Analysis and Molecular Weight Determination

A 0.05% solution of the lyophilized fibrin monomer was prepared in buffered saline. The sample was run using a Water's 510 isocratic pump at 1 ml per minute using buffered saline as the mobile phase. Size exclusion chromatography was performed using a Shodex 8 mm x 300 mm 7 mm KW-803 column using Water's 450 UV detector; detection was performed at a setting of 280 nm.

In order to determine the molecular weight range of the fibrin monomer a calibration curve was prepared using water soluble proteins having known molecular weights. Standard 0.05% concentrated solutions of Blue Dextrin (2000K), g-Globulin (150K), Bovine Serum Albumin (66.3K) and Myoglobin (17.6K).

Figure 2 shows the calibration curve generated using these standards. The relative molecular weight was single modal and determined to be 346K Daltons when prepared using 250 units of thrombin to 100 mg of fibrinogen. When 125 units of thrombin was used to prepare the fibrin monomer bimodal molecular weights were evident. These were estimated to be 350K and 316K. The former comprised approximately 65% of the sample and the remaining 35% was attributable to the 316K molecular weight fraction.

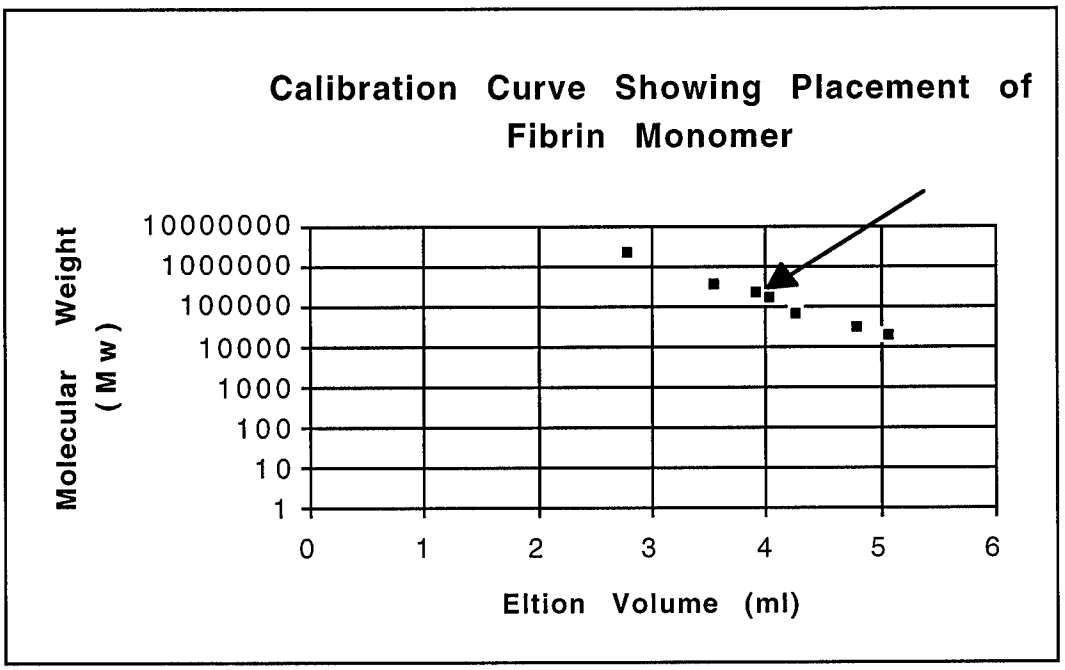

Figure 2. HPLC molecular weight calibration curve.

Further characterization was required to determine whether thrombin concentration played a role in the integrity of the formed fibrin clot. In order to examine these effects, the amount of thrombin were varied from 1, 10, 50, and 500 units.

Fibrin monomer was prepared by mixing stochiometric amounts of fibrinogen and thrombin (in the experimentally varied amounts), the subsequent solution was then mixed utilizing a gentle agitating motion. The soft gel that formed was dissolved using a 10% ammonium hydroxide solution. The soft gel was placed in a bell jar, and a vacuum was applied to remove the ammonia, allowing the remaining solution to be frozen. The frozen solution was placed in the lyophilizer and dried. The white powder that was formed was visually inspected and then a sample removed for HPLC analysis.

The percent yield after drying was determined to be 79.6%, 88.6%, 80.1%, and 75.6% for thrombin concentrations of 1, 10, 50, and 500 units respectively. Figure 3 is a graphical analysis showing the results of the various thrombin formulations and their relative effect in regards to the amount of clottable protein.

The molecular weight and composition of the fibrin for the four formulations were determined to be as follows: the 500 unit formulation was composed of 45% 288K and 55% 129k; the 50 unit of 24% 249K and 76% 82K; the 10 unit of 100% 277K; and the 1 unit formulation was 100% 305K.

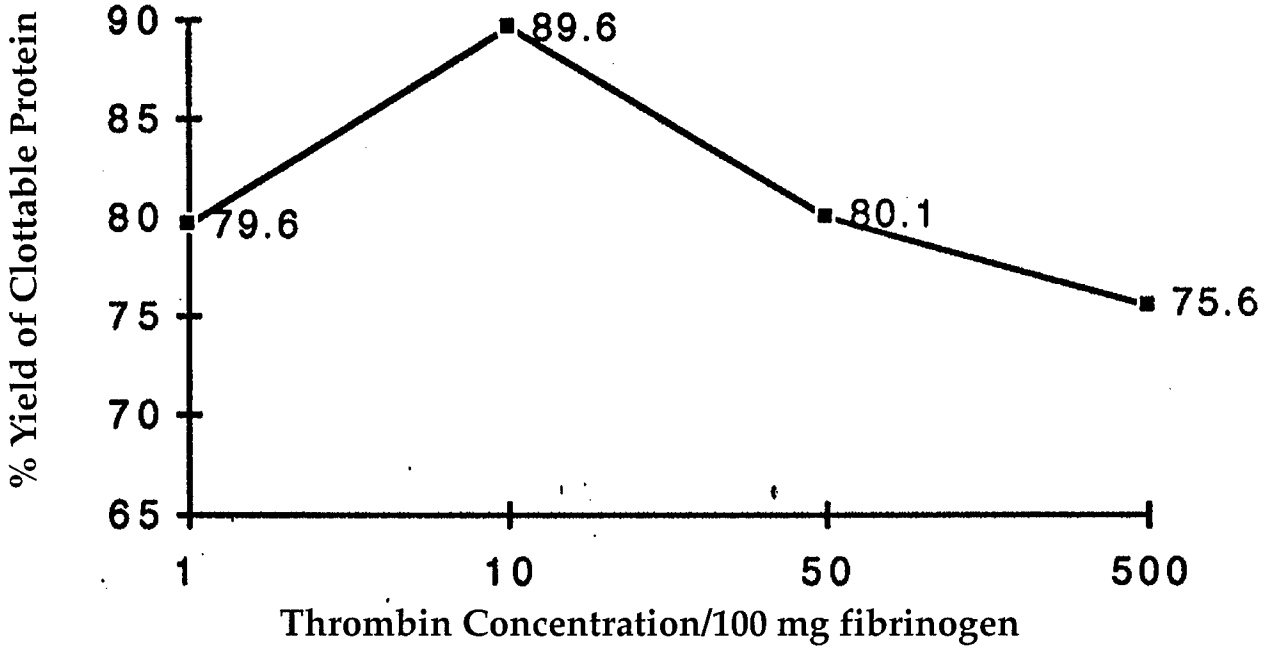

Figure 3. Fibrin yield versus thrombin concentration. The maximum yield occurs with a throbin concentration of 10 NIH units/100 mg of fibrinogen

Process Development of the Fibrin Formulation

Each of the constituents were individually produced and analyzed with HPLC to acquire characteristic "signatures" of the components. These were used later to identify the constituents when placed together in solution.

The HPLC analyses were performed with the use of a Waters HPLC model 510 isocratic pump with a Shodex protein KW-803 column (8 mm x 300 mm). The packed columns of the Shodex protein KW-800 series are designed to be used in high performance chromatography for separating proteins, enzymes and polysaccharides. The mobile phase was 0.9% NaCl and a flow rate of 5 ml/min was generated with a Waters Solvent Delivery Module (Model 590). Fifty microliter (50µl) injections of the sample were introduced through a Waters U6K injector.

The amount of sample present was quantified by means of a Waters 450 Tunable UV Absorbance Detector, set at 250 nm and 1.0 AUFs, connected to a Waters Data Module (Model 730). The Data Module is a microprocessor based printer/plotter/integrator designed to provide quantitative information for HPLC applications.

The mobile phase was characterized by injecting a 50 ml sample of the 0.9% NaCl solution. After acquiring the characteristic pattern of the saline, the testing of other agents could proceed.

Solutions of the above mentioned constituents were created for characterization as follows. Thrombin (Thrombostat®, Parke-Davis, Morris Plains, NJ) 20,000 NIH units /20 ml isotonic saline, was compounded to provide a concentration of 1 mg of thrombin per 1 ml of 0.9% sodium chloride. Fibrinogen (fibrinogen fraction 1 Type IV: from bovine plasma, Sigma Chemical Company, St. Louis MO.) was reconstituted to provide a concentration of 1 mg of fibrinogen per 1 ml of 0.9% sodium chloride. Aprotinin is available in solution (Trasylol®; Miles Inc., West Haven, CT) and was used directly.

Fibrin monomer was obtained from the products of the reaction between fibrinogen and thrombin. As in the final stages of the coagulation cascade, the plasma protein, fibrinogen, is cleaved by the protein enzyme, thrombin. Fibrinogen is a symmetrical molecule containing three pairs of non-identical peptide chains, the overall structure being (Aa, Bb, γ)2. The chains are held together by disulfide bridges. The proteolytic attack of thrombin on fibrinogen is highly specific and limited. Thrombin cleaves an arginyl-glycine bond in each of the a and b chains, releasing two A peptides and two B peptides.

The remaining portion of the molecule is termed fibrin monomer. The fibrin monomer spontaneously polymerizes with other fibrin monomer molecules, forming long fibrin threads within seconds. Therefore, such a reaction will produce a fibrin coagulum.

To accomplish this, the fibrinogen and thrombin solutions were combined in a 1:1 ratio and allowed to coagulate. The resulting formulation was then centrifuged and the supernatant decanted. The supernatant was saved to obtain the signature of the cleaved

fibrinopeptides.

The remaining fibrin coagulate can be dissolved into soluble fibrin monomer with the use of urea, high salt concentrations, or pH extremes. This is because the early stages of polymerization consists of hydrogen and hydrophobic bonds which can be broken apart with relative ease. To isolate the fibrin monomer, the fibrin coagulum from the above example was dissolved in a solution of 8 M urea.

A solution of 8 M urea and a 0.1 M acetic acid will dissolve the coagulated fibrin, thus releasing fibrin monomer. However, subsequent lyophilization of the solution resulted an inseparable mixture of fibrin monomer and urea crystals. Consequently, the fibrin monomer was dissolved in a solution of ammonium hydroxide. Lyophilization of this solution resulted in the evaporation of the ammonium hydroxide, leaving the fibrin monomer behind as a white powder. The monomer was reconstituted in saline solution for characterization with HPLC methods.

These solutions of the hemostatic agent constituents were analyzed with HPLC to ascertain the characteristic signature of each. Fifty microliter (50 µl) samples of each solution were injected for chromatographic analysis. Blanks of the reconstituting solutions were also run to verify their presence. This procedure was repeated three times to assure accuracy. Consequently, the following constituents were identified with HPLC methods: thrombin, fibrinogen, aprotinin, saline, fibrinopeptides A and B, and fibrin monomer. These were used later to confirm the composition of the agent prior to lyophilization.

Our method for preparing fibrin nonomer is as follows: Lyophilized fibrinogen purchased (Sigma Chemical Company, St. Louis, MO) is combined with thrombin (Thrombostat, Parke Davis or equivalent). The mixing ratios are **shown in Table 1.**

Table 1. Main ingredients of the fibrin monomer based hemostatic agent.

fibrinogen	110 mg
water	1 ml
reconstituted thrombin	10 NIH units
ammonium hydroxide (10%)	10 ml

Final Fibrin Solution Production/Manufacturing Process

To prepare the hemostatic agent, 110 mg of fibrinogen is first dissolved at room temperature in distilled water and sonicated briefly, if necessary. Thrombin is similarly rehydrated. The 10 units of thrombin are injected into the fibrinogen solution, and the two react quickly to form a soft gel.

A 10 ml volume of 10% ammonium hydroxide solution is added to this gel, and this mixture is sonicated or agitated until the gel dissolves. The dissolved fibrin monomer can

then be lyophilized or it can be immediately dispersed into a solution. The fibrin monomer solution is stable for up to 2 weeks if refrigerated at 40 C.

Sterilization of Final Fibrin Solution.

A suitable method of sterilization of the final lyophilized powder form of the fibrin monomer was then needed. A solution of the lyophilized fibrin monomer was prepared according to protocol. Two batches of fibrin monomer were made using 10 units of thrombin to 100 mg of fibrinogen for gamma irradiation experiments. Approximately 85 milligrams of product were recovered after dissolving in ammonia and filtering with a 5 μm filter. The resultant clot was then lyophilized overnight and washed with distilled water and filtered through 10 μm filter. The remaining slurry was then dried in a vacuum, weighed, and separated into two parts consisting of 40 mg and 45 mg respectively.

The powder was then divided and placed in quarantine for later comparison with the sterilized amounts. One portion of the lyophilized powder was placed in a test tube and irradiated with 2.5-5 megarads of gamma radiation. The powder was returned to the laboratory and tested. HPLC results of samples sterilized by gamma irradiation were compared to control samples from the same lot. Results showed a slight shift in the elution time in the sterilized samples but the peak shapes were similar for both the control sample and the irradiated sample.

HPLC results using the same methodology obtained for the fibrin monomer samples sterilized by gamma irradiation were compared to results for control samples from the same lot. First attempts using a single reversed phase μBodapak C-18 column (3.9 X 150 mm) indicated that both the control and the irradiated sample exhibited more than one peak. Detection was performed at a wave length of 206 nm using 2.0 ABS units and a column loading of 25μg of fibrin monomer.

Attempts at improving peak resolution by changes in the mobile phase were unsuccessful, but better resolution was obtained using two columns in series utilizing the above operating parameters. Figure 3 shows typical chromatograms of both the control and the gamma irradiated samples. The control shows two differentiated peaks at retention times of 2.23 and 2.45 as well as the appearance of an emerging shoulder. The irradiated sample shows three separate peaks at retention times of 2.16, 2.56 and 2.91.

This data appears to indicate that gamma irradiation alters the chemical structure of the fibrin monomer, but whether such changes may significantly affect the properties of the fibrin monomer is not discernible by this analysis. No attempts were made to identify the constituents of the irradiated sample or compare them with known fibrinolysis products, as it seems unlikely that the denatured protein would retain its normal activity and function. Batch sterilization using this method is thus not applicable.

We therefore rely on sterilization by filtration of the solution before lyophilization. This requires that all subsequent processing be done under sterile conditions.

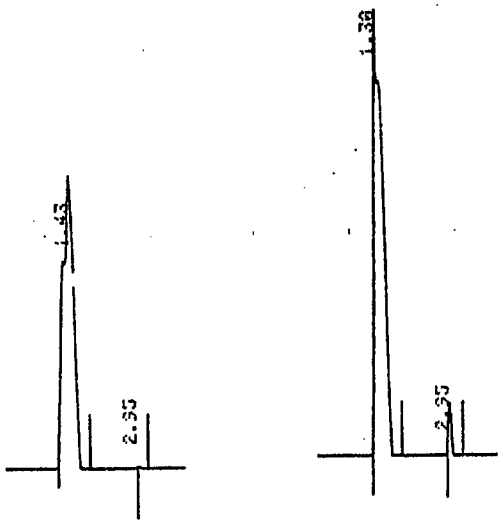

Figure 3. Chromatographs of non-irradiated fibrin monomer on the left and gamma sterilized fibrin monomer on the right. This method of sterilization appears to denature the protein.

Task II. Chronic Tissue Healing Studies

In this task, we performed tests of the fibrin monomer agent to produce hemostasis in vivo using a splenic injury model, and we then examined the tissue response to the material for healing duration's extending to 1 month. We also used those animals at sacrifice to test the ability of the hemostatic agent to control arterial bleeding.

Splenic Injury Model Tests

In vivo tests conducted at the Cleveland Clinic Foundation to measure the ability of the fibrin monomer based hemostatic agent to control bleeding provided remarkable results. The new agent was compared directly with Tisseel®, the fibrin tissue adhesive currently available commercially in Europe and the far east. The fibrin monomer agent produced hemostasis significantly faster than Tisseal®. This difference was statistically significant ($p<0.001$). A summary of this testing follows.

The basic fibrin monomer hemostatic agent (F), the fibrin monomer hemostatic agent containing aprotinin (FA), and Tisseel® were tested for their effects on hemostasis and tissue adhesion using the splenic controlled trauma model in rabbits. Parallel sharp cut wounds, 7 mm long and 2 mm deep in size, were made on the splenic capsule and into the parenchyma with a #15 blade equipped with specially designed incision length and depth guides. Each hemostatic agent was applied to a wound site, and gentle compression was applied with a gauze sponge for the first three minutes. Hemostasis was checked every minute thereafter by gently removing the gauze sponge. The time required to obtain complete hemostasis was recorded as the bleeding time.

Table 2 depicts the bleeding time measured from all 36 cases. As a negative control,

wound #4 was tested without any hemostatic agent, and it continued to bleed for more than 60 min. Because of this, the bleeding time for the wound #4 was measured in only four cases.

Table 2. Measured Bleeding Time (min,) Splenic Injury Model in Rabbit

Experiment#	Wound #				#1(F)	#2(FA)	#3(Tisseal)	#4(Gauze)
96009	3	7	15					
96010	3	13	26					
96011	5	3	60		>60			
96012	3	3	70		>70			
96013	3	6	13					
96014	5	4	15					
96025	3	3	16					
96026	4	22	58					
96028	3	5	14					
96029	6	4	10					
96037	3	4	32					
96038	4	10	8					
96039	3	4	15					
96043	3	3	13					
96044	5	5	10					
96045	4	4	13					
96049	3	3	7					
96050	7	10	11					
96051	3	3	10					
96052	3		3	12				
96062	3	3	30					
96063	4	4	68					
96064	3	6	46					
96068	4	5	10					
96069	4	4	15					
96070	3	6	48					
96073	3	3	9					
96074	3	6	10					
96075	3	3	10					
96076	3	3	56					
96077	3	3	15					
96078	20	10	29		>60			
96084	3	3	8					
96085	3	4	15					
96086	3	20	50					
96087	3	3	23		>60			
Ave.	4.00	5.69	24.17		>60			
SD	±2.91	±4.50	±19.21					

Both "F" and "FA" showed a significantly shorter bleeding time than Tisseel®, and the differences were statistically significant to $p<0.001$.

It was also noted that Tisseel® was more easily removed from the wound surface when the gauze sponge was elevated for inspection, possibly explaining occasional prolonged bleeding times recorded (#'s 96011, 96012, 96026, 96063, 96076, and 96086). In contrast, fibrin monomer preparations F and FA appeared better adherent to the wound edge and surface, as judged by the continued presence of whitish fibrin clots around the wound. A total of 36 rabbits were used to observe the trauma sites both macroscopically and microscopically after 2 days (n=12), 1 week (n=12), and 1 month (n=12).

The animals recovered from surgery uneventfully, and there was no occurrence of hemoperitoneum or recurrent bleeding. The abdominal surgical wound healed well. A mild, thin fibrous adhesion was observed in 13 of 34 cases, between the visceral organs or the omentum and the peritoneum of the laparotomy site. In 30 of 34 cases, thin fibrous adhesions were noted between the spleen and the surrounding tissues, mainly with the omentum and the small intestine. The adhesions, however, were mild and bluntly dissected with ease. **No hematomata or sizable granuloma suggestive of preceding hematoma formation was noted. The wounds were covered by whitish fibrous caps, which decreased in amount with time.**

Cross-sections for histology were obtained from the mid-portion of the wounds, fixed in HistoChoice, sectioned and stained with hematoxylin and eosin (H&E) and Masson's trichrome. The fibrin monomer formulations and Tisseel® were recognized as eosinophilic and amorphous substance filling the typically "V" shaped wound and covering the adjacent splenic capsule. Two fibrin monomer preparations appeared to be slightly more eosinophilic. After 2 days, there was still a small amount of accumulation of erythrocytes(RBC) along the wound edge. Hemosiderin granules were rather conspicuous. Migration of polymorphonuclear leukocytes (PMN) was mild. The proportion of eosinophil appeared to be increased. The number of mononuclear leukocytes(MN) was still small. A small number of fibroblasts and collagenization were recognized along the wound edge. Acute inflammatory response, thus, seemed to be mild in all three hemostatic agents tested.

By one week, absorption of fibrin monomers and Tisseel® was more obvious, with an increased number of MN and an occasional appearance of foreign body type giant cells surrounding fibrin monomers and Tisseel®. Fibrosis was further advanced in the peripheral regions.

By one month the wound healed in the majority of the cases with a small amount of fibrous scar tissue, with mild chronic inflammatory responses. When fibrin glues or Tisseel® persisted they are trapped in the granulomatous lesions composed of MN, fibroid cells and occasional giant cells. A more quantitative comparison of these three hemostatic agents was attempted, comparing the cross sectional area of the wound and its change with time and the degree of inflammatory responses.

The difficulty encountered in that quantitative analysis was that the amount of applied

hemostatic agent was not able to be controlled. Thus, the degree of inflammatory response and the rate of healing of each wound may reflect the amount of foreign materials topically applied, and not the inflammatogenicity or immunogenicity of the material tested.

One peculiar observation has been that Tisseel® is occasionally delaminated from the splenic capsule as evidenced by the presence of fibrin clots between the fibrous capsule of the spleen and the Tisseel® layer. This may support our observations during bleeding time measurements that the adhesiveness of Tisseel® to the splenic capsule was not as strong as those of the two fibrin monomer formulations.

A complete summary of the histological findings with illustrative photomicrographs is contained in Appendix A.

Task III. Delivery System Development and Testing

The objective of this task was to integrate the new hemostatic agent into a complete, pre-packaged wound dressing system which will readily allow it to be used under battlefield conditions. This system includes two distinct types of delivery modes to be used separately or in combination depending on the wound characteristics.

One is open-cell, hydrophilic foam sponge designed to serve as a carrier for the fibrin monomer based hemostatic agent. It provides a delivery system which is intended for controlling bleeding with deep tissue injury, possibly including arterial bleeding.

The other is an elastic, semi-occlusive dressing incorporating the hemostatic agent designed to control bleeding while covering and protecting the site of superficial injury and releasing an antimicrobial agent to help control infection. Together, these dressings will provide superior wound stabilization and treatment.

Polymer Foam Testing and Development

Previous reports have described two separate methods for the preparation of polyurethane foams suitable for use in this application. The first employed the single shot method where all the reagents were mixed together and cast into a mold. The second method required the preparation of a prepolymer followed by further polymerization which was controlled by the addition of a chain extender.

Further testing was performed to define an appropriate open cell foam as a method of delivery for the tissue adhesive/hemostatic agent. The results of these tests indicated that the single shot method would provide the better vehicle from which to launch the production of the foam carrier. In order to reduce the chance of batch-to-batch inconsistencies, it was decided to prepare one large block of foam prepared from the same lots of raw materials. Utilizing the single shot method, a foam sample large enough to provide sufficient samples for toxicity testing was made. This operation required the preparation of sheets totaling

3000 cm2 in area.

The following reactants mixed at their stated weight ratios. First 580 gm of the polyol Pluracol L64 was added to a polypropylene cup having sufficient capacity to hold the liquid foam reactants. To this was added 12 gm water and 2 gm of catalyst T12. These three reactants were mixed with a mechanical stirrer and set aside for later use. In a separate polypropylene container 248 gm of Mondur CD was added. Both containers were allowed to equilibrate to ambient temperature 250C.

The container containing the polyol was replaced under the mechanical stirrer and the Mondur CD was added with rapid agitation. The mixture had a 45 to 55 second reaction time. The stirrer was removed and the rising liquid was cast into a polyethylene lined rectangular mold, pre-heated to 37^0C. The foam was allowed to cure at this temperature for 3 hours.

The following day the foam block occupying approximately one cubic foot, was removed in preparation for physical testing and cutting of toxicity samples. The foam block was cut into twenty sheets approximating 7 cm. x 22 cm x 0.5 cm and the remainder set aside. These sheets were dialyzed in buffered saline for 48 hours, with the solution changed at the 24 hour mark. The dialyzed samples were patted dry and placed in a 50^0C oven for one hour to dry.

Toxicity testing was then performed with this material. Those tests are described in detail in a later section of this report. The foam passed all USP Class VI tests, and samples of the foam with the fibrin based hemostatic agent were prepared to test the effectiveness of the foam as a delivery system. Figure 4 is a photograph of the open cell foam with the hemostatic agent applied on its surface. The presence of the agent is barely discernible.

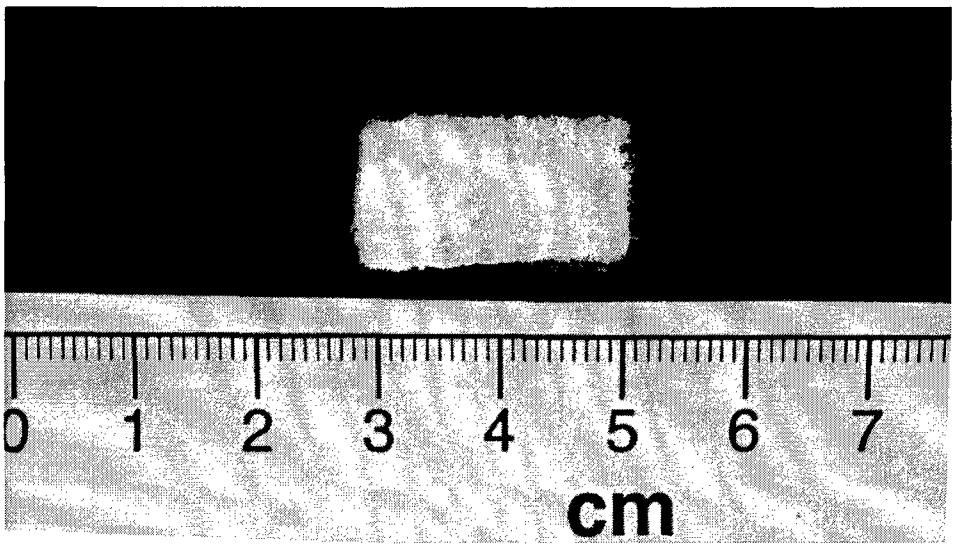

Figure 4. A photograph of the open cell foam used as a delivery system with the hemostatic agent applied to its surface. This delivery method minimizes the amount of material used. The foam also provides some mechanical compression and absorbs water to concentrate clotting factors.

In Vivo Testing of the Hemostatic Sponge Delivery System

The open-cell hydrophilic polyurethane foam was designed to serve as a carrier for the hemostatic agent for treating deep tissue injury with possible arterial bleeding. The sponge serves several functions: (1) it provides compression by virtue of its elasticity; (2) it delivers the hemostatic agent as directly to the site of bleeding because it conforms to the shape of the tissue; (3) it absorbs water, serving to concentrate clotting factors to help achieve hemostasis; and (4) it minimizes the amount of the hemostatic agent used.

Previously available hemostatic agents often have been packaged as loose powders. Unfortunately, there means that there is a tendency to use amounts in excess of that actually required. That excess material must be metabolized during healing, and this prolongs inflammation and slows the healing process. By placing the fibrin monomer agent on the surface of the sponge, the amount of material used is minimized, and this alone will promote faster healing. The agent is not bound to the inert polyurethane surface. Thus, when activated by blood and placed in proximity to tissue, the hemostatic agent bonds to the tissue and freely releases from the sponge surface.

The hemostatic sponge was tested using the rabbits from the splenic injury study. Eight male New Zealand white rabbits were studies immediately before sacrifice. The surgical procedure consisted of exposing and transsecting the left and right superficial femoral arteries, vessels approximately 1 mm in diameter. The hemostatic sponge with the hemostatic agent on its surface was applied to the left femoral artery, while the sponge material without the hemostatic agent was applied to the right femoral artery and used as a control. Gentle compression was maintained for the first 3 minutes, and the wounds were checked. Subsequently, the wounds were checked every minute thereafter. The time that was required for each wound to obtain complete hemostasis was recorded as the bleeding time.

The results are shown in Table 3.

Table 3. Measured Bleeding Time (Minutes)

Experiment #	Hemostatic sponge	Control Sponge
96074	5.0	8.0
96076	7.0	23.0
96077	5.0	6.0
96078	4.0	20.0
96084	9.0	26.0
96085	13.0	26.0
96086	8.0	8.0
96087	12.0	10.0

The mean bleeding times for the sponge with the hemostatic agent versus the controls were 7.9 ± 3.3 min and 15.9 ± 8.7 min respectively. The sponge with the hemostatic agent produced significantly shorter bleeding times ($p>0.028$). This suggests that the hemostatic agent applied to the sponge is likely to be useful in controlling arterial bleeding.

Development of the External Wound dressing

The primary use and design of the external dressing is to be for emergency conditions where immediate stabilization of the individual awaiting evacuation is requisite. Therefore, the initial objective of the dressing is to control bleeding, then to prevent infection by controlling local bacteria with the use of antimicrobials.

Traditional wound dressings have generally consisted of sterile, absorbent cloth pressure bandages. These materials have minimal beneficial characteristics and primarily function as simple coverings to protect wounds. These dressings provide little protection from infection. Indeed, they can provide an environment conducive to bacterial proliferation. By being absorbent, these dressings can dehydrate the wound. Since the materials of these dressings are not impervious to microorganisms, the saturated dressing may provide an excellent substrate supporting microbial growth. Consequently, infection is potentially aided rather than prevented.

This situation can potentially be ameliorated by the incorporation of antibiotics in the dressing to provide aseptic conditions at the wound surface.

The external wound dressing consists of four layers: (1) a top layer consisting of an elastomeric film which acts as a protective barrier; (2) an adhesive layer to hold the dressing on the skin ; (3) a silicone island which contains a timed release antibiotic; and (4) a coating on the tissue facing surface consisting of the hemostatic agent.

The development of the barrier film was not included in this program, as many commercial films already exist which are suitable.

Adhesion to Skin

Skin adhesion is a fundamental property required to hold any device in place. However, adhesive properties should be such that the dressing can be removed after the required residence time in an unremarkable manner. Skin adhesion should be balanced between: (a) the adhesion level required for secure holding regardless of patient movement, perspiration level or bathing, and (b) ease of removal when dosage is complete.

The most desirable adhesive system will also show uniform adhesion to skin over time with only moderate adhesion buildup or loss. Also, the range of values observed should be statistically reproducible and as small as possible.

Adhesion of a wound dressing should only be enough to effectively keep the device in place for the necessary dosage period. Higher levels of skin adhesion should be avoided

where possible, since high skin adhesion levels increase the incidence of excoriation during removal. Higher than necessary levels of skin adhesion also increases the probability of skin sensitization and irritation with repeated use on the same site.

In our medicated wound dressing, we have chosen an acrylic-based, pressure-sensitive adhesive that builds adhesive to the skin site rapidly, plateaus, and thereafter maintains uniform adhesion for up to seven days. Upon removal, we have observed a minimum to adhesive residue left on the skin site, and removal has been unremarkable.

Cohesive Strength

This is the ability of the adhesive to stay together and to stay in place under load, i.e. resist shear. Good cohesive strength is also vital for clean removal from the skin with minimum residue. It is a manifestation of the visco-elastic properties of a particular system.

Cohesive strength is a function of the molecular weight and molecular weight distribution. Addition of relatively low molecular weight tackifying agents to compounded adhesives affects the molecular weight distribution. Adhesive processing during coating can also directly influence final molecular weight distribution.

Positive tests for good cohesive strength in vivo are the unit staying in place on the patient (not sliding) and unremarkable removal with no visible adhesive residue left on the skin. In our wound dressing, we selected a pressure-sensitive adhesive which displayed sufficient cohesive (or internal) strength to remain in place, yet it peeled from the skin cleanly.

Anchorage of the Adhesive

The pressure-sensitive adhesive, which is designed to hold a medicated wound dressing to a soldier's skin, must on the other side stay adhered to the dressing. Keeping the adhesive firmly attached to the dressing is referred to as adhesive mass anchorage.

Adhesive mass anchorage is most easily tested in a direct manner. The tests are essentially qualitative; either it is satisfactory or it is not. An effective test that can be done without instrumentation is to simply fold the adhesive film composite pressure-sensitive side upon itself and press together to ensure good contact. Then peel one end back on itself creating a 180^0 peel test. Outcomes other than clean separation constitute failure.

Adhesive Qualification

Most commercial polymer dressings are composites of thin urethane films coated on one side with a medical grade pressure-sensitive adhesive. The advantages of these composites are their light weight, conformability and high moisture vapor transmission rates(MTVR) while still remaining occlusive to water and bacteria. The choice of acrylic pressure sensitive adhesive over other polymers is based upon the fact that these adhesives

are commercially available in medical grades and that these adhesives are hydrophobic and can maintain their adhesive strength after immersion in water.

Adhesive transfer tapes manufactured by Adhesive Research Inc., under the brand names ARCARE 7560 and 8311 have shown they may be a likely candidates. These medical grade adhesives have typical MTVR's of 500 to 1000 g/m^2/day respectively, and been shown to retain high adhesive properties even after immersion in water for 72 hours. Twelve test samples were prepared by bonding the adhesive coated polyurethane membrane to test strips prepared from sheep's skin, using both ARCARE adhesive composites. Six samples were maintained at ambient conditions, the remaining six samples were immersed into water and conditioned for 72 hours. Peel tests will be performed using the test assembly shown in Figure 3 per ASTM D-930 procedure. Results of wet and dry samples were compared and the percent retention determined.

Results

The group using the Arcare 7560 showed the highest peel strength for both dry and wet samples. This group did loose 50% of its dry peel strength after exposure but the immersed samples still exhibited high peel strengths of 27 g/cm. All samples were listed as adhesive failure to the leather substrate.

The Arcare 8311 showed an increase in the peel values for the wet samples compared to the dry ones. But, the absolute values compared to the 7560 80 to 50% lower. The Arcare 8311 also exhibited adhesive failure to the urethane film as opposed to adhesive failure to the leather substrate. This data is shown in Figure 5.

Arcare	g/cm	Average	Trial# 1	Trial# 2	Trial# 3	Trial# 4	Trial# 5	Trial# 6
7560	Mean	54.7	38.7	55.0	53.3	62.0	57.7	61.4
Dry	SD	7.7	11.6	8.1	7.1	4.6	11.0	3.8
	Max	62.4	48.6	61.4	59.1	66.8	73.8	64.5
	Min	36.2	33.8	30.4	32.1	47.5	29.1	44.2
Arcare								
7560	Mean	27.3	26.4	29.8	31.2	22.4	25.6	28.3
wet	SD	9.0	8.0	9.8	11.2	7.2	8.3	9.4
	Max	40.7	39.5	44.3	43.1	35.6	38.7	42.7
	Min	11.7	10.3	11.9	1.7	8.2	29.1	8.8
Arcare								
8311	Mean	10.1	8.4	7.0	11.3	11.2	11.7	11.1
Dry	SD	0.9	.2	.3	1.8	.5	1.1	1.2
	Max	11.6	9.0	7.4	16.2	11.9	12.6	12.3
	Min	7.2	8.0	6.3	8.2	9.5	5.0	6.5
Arcare								
8311	Mean	14.5	15.5	14.3	17.0	14.3	13.3	12.8
Wet	SD	1.2	.7	1.4	1.3	1.3	.9	1.6
	Max	16.3	16.3	15.9	18.3	18.3	14.5	14.2
	Min	9.6	11.9	8.0	11.3	11.3	8.4	6.7

Figure 5. Summary of Peel Tests for Dressing Substrate.

On the basis of these tests, the Arcare 7560 adhesive system was then used to fabricate the external wound dressings which were employed at the CCF to conduct the following evaluation.

Tests of the External Wound Dressing System

The external wound dressing was tested using a series of 12 female pigs (22.6 ± 3.9 kg). A full thickness skin incision , 2 cm long, was made bilaterally on the abdominal and dorsal regions using a #15 scalpel. The left side wounds were treated with the test dressings, while the right side wounds were treated with surgical skin staples. Skin blood flow at the wound sites were measured using a laser Doppler flowmeter. Animals were sacrificed at 1 week (n=6) and 1 month (n=6). Test specimens were taken from the wound sites for wound tear strength measurements and histological analysis.

Figure 6 is a photograph of a wound site immediately after the dressing was applied. The release of the hemostatic agent from the dressing appeared to be effective in preventing bleeding. After 1 week, the chief difference between the wound dressing treated wounds and those treated with surgical staples was reduced inflammation.

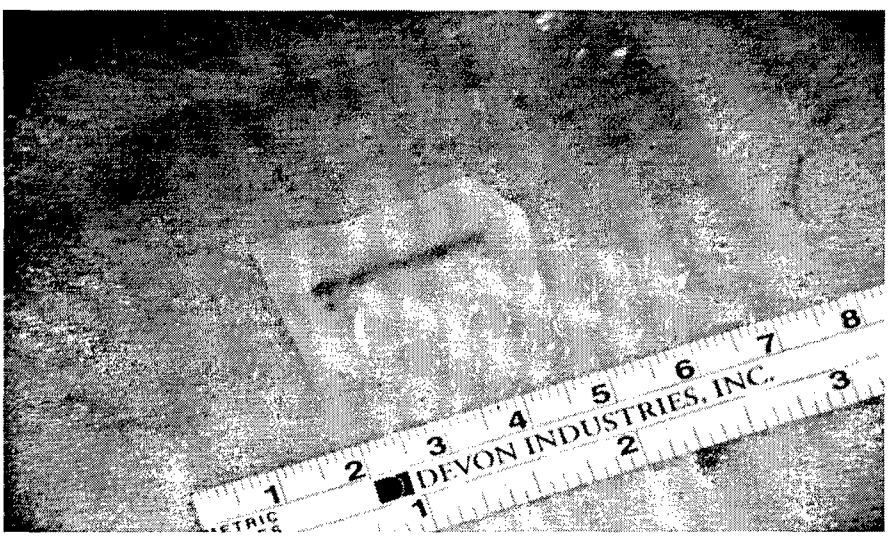

Figure 6. The hemostatic wound dressing on day 1. It provides approximation of the tissue margins and release of the hemostatic agent to control bleeding and bond the tissue together.

At 1 week, the wound tear strength measurements were:

Rt-abdominal	0.391 MPa ± .403
Lt-abdominal	0.257 MPa ± 111
Rt-dorsal	0.172 MPa ± .086
Lt-dorsal	0.310 MPa ± .189

There is no significant difference in the wound strength at either site at this duration. Thus, the external wound dressing with the tissue adhesive was as effective as skin staples in providing wound closure·

At 1 month, the wound tear strengths were:

Rt-abdominal	4.756 MPa ± .771
Lt-abdominal	1.660 MPa ± 681
Rt-dorsal	4.793 MPa ± 1.369
Lt-dorsal	4.013 MPa ± 1.321

With this duration, there is no difference in the tear strength of the dorsal wounds, but a difference was noted between the left and right abdominal wounds ($p<0.005$). We speculate that the abdominal skin in this model is subject to more stress than the dorsal skin because the animal lies on that surface, and that staples are better able to maintain the tissues in contact over the full skin thickness in that situation.

Tissue healing thus occurred at comparable rates and strengths with the exception noted. No infection was noted histologically in either group. The advantage of the wound dressing was that it produced no acute inflammatory reaction, an effect which appears inevitable with the staples. This is illustrated in Appendix A.

For battlefield use, the external wound dressing with the hemostatic agent has the potential to be an easily applied device to protect the injury site, approximate the edges of damages tissue, and provide healing with a wound strength comparable to surgical skin staples. As it also contains a timed release antibacterial agent, it may also aid in reducing the incidence of infection, but that was not studied directly in this program.

Task IV. Controlled Release of Antibiotics

Although hemostatic agents are designed for immediate activation, pharmacologic agents may be useful both immediately, to control initial infection, and over time, to prevent further infection. Therefore, antimicrobials may be present in both non-encapsulated and/or encapsulated forms for immediate release and sustained release, respectively.

Sustained or controlled release of drugs, or any additive, has been investigated for many years. The most mechanically simple and smallest release devices are those consisting of polymer matrices. Here the release rates are determined by the diffusion rates of the additive in the polymer or, as in a bioerodable system, by the degradation rate of the polymer. In many situations, combinations of both occur simultaneously. So once the additive has been selected, the polymer choice is of utmost importance. These polymers must be non-toxic, able to be processed into usable matrices by familiar methods, and must not alter the additive function. Three common polymers used for controlled release systems

are poly(DL-lactide co-glycolide) or PLGA, polymethylmethacrylate, and polycyanoacrylates. All of these however, are brittle, degrade slowly, or have effective release periods much different than those needed for a short term dressing (one week maximum). We thus concentrated on developing a flexible polymer film suitable for a dressing which will have a well defined elution rate over a period of at least 1 week.

Antibiotic Selection

Meaningful statistical data from soldiers in the field with combat casualty infections are rare. This is understandable, since in wartime there is little time to conduct statistically designed bacteriologic surveys. Wound infections are customarily treated without culturing, and only those infections that prove resistant to conventional therapy are ultimately cultured.

Infections in the field will typically involve a mixed flora. Bacterial contamination in field wounds typically involves normal skin flora, dirty clothing and sweat contaminants, fecal soiling contaminants, various soil organisms depending on geography and region, other resident environmental flora, and organisms found in standing water (many gram-negative organisms; i.e., Pseudomonas, Serratia, enterobacteria, Herellea, Flavobacterium).

Antibiotic-loaded field wound dressings must be capable of controlling bacterial infection in contaminated wounds, or be able to control established infections such as those encountered in dirty and infected wounds. The proliferation of fungi primarily occurs in the absence of local bacteria. However, it is unlikely that this will occur in the time span that the dressing is to be worn. Therefore, a dressing containing a single antimicrobial should provide ample protection until the individual is given professional medical aid.

We selected chlorhexidine gluconate (CHX) as the antimicrobial for the dressing. Chlorhexidine is a cationic biguanide microbicide with a broad spectrum of activity against many forms of bacteria. Specifically, chlorhexidine acts to disrupt the permeability of the bacterial cell membrane leading to lethal destruction. We have been using this drug with some success in other devices being developed in our laboratory (such as a Foley catheter incorporating CHX to help prevent the development of urinary tract infections in patients with indwelling catheters.

In Vitro Antibiotic Release Studies

The release of the antibiotic from the material over extended time periods was also examined using HPLC. These tests measured the amount of antibiotic released over a 24 hour period up to a duration of 2 weeks. The measured release is shown graphically in Figure 7. Over this duration, sustained release of chlorhexidene occurs at levels sufficient to suppress bacterial growth. The next step was to conduct tests of the bactericidal capability of the antibiotic loaded silicone substrate.

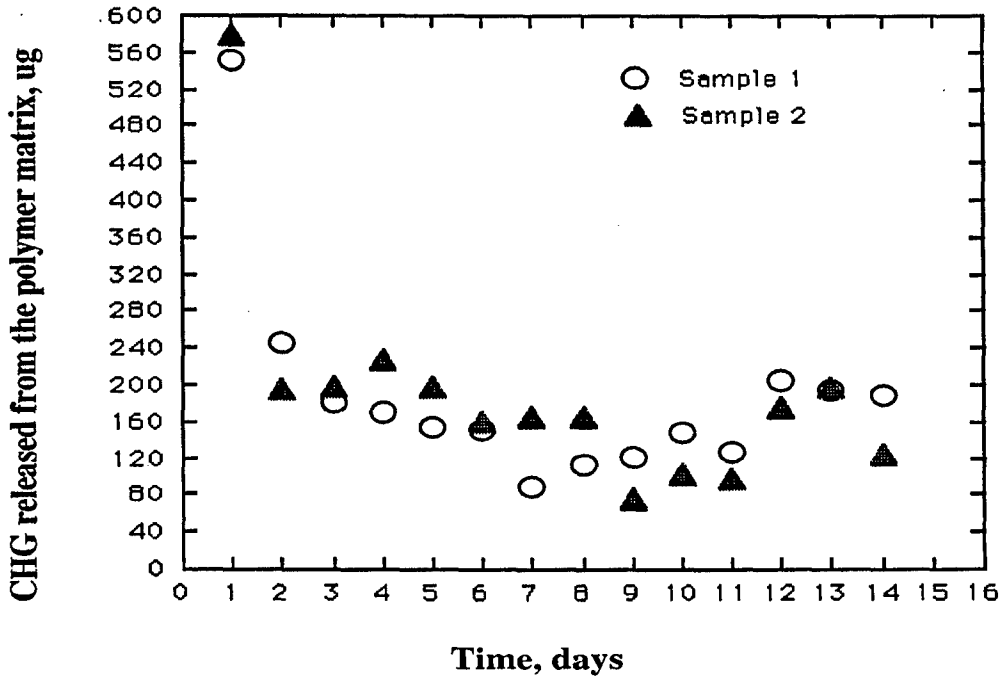

Figure 7. Measured CHX release from the silicone rubber substrate of the external wound dressing. The drug is released by diffusion, and its release is sustained over an extended period.

Bactericidal Activity Studies

To test the efficacy of antibiotic release from the silicone substrate, zone inhibition tests were conducted by Microbiology Research Associates (Acton, MA). Silicone rubber disks loaded with 0, 1, 2, and 3% CHX were prepared. These were then used to conduct zone inhibition tests with three microorganisms: P. aeruginosa, S. aureus, and S. pyogenes. The complete results from this testing are provided in Appendix D.

The methodology used was as follows. A test sample consisting of 0.5 ml of a 1:1,000 dilution of test organisms in phosphate buffer was added to the bottom of a sterile empty petri dish and a negative control dish. Separately, each test disk was then placed directly on top of the 0.5 ml sample of the test organisms to create a sandwich effect. Each disk was then allowed to have direct contact with the microorganisms for 1 min., 5 min., 30 min., and 1 hour at room temperature.

After the contact time interval, each disk was asceptically placed in a sterile empty tube. The remaining 0.5 ml of the test sample was pipetted into the tube with the disk, and the petri dish was washed with 9.5 ml of neutralizing buffer and added to the tube containing the disk. The contents of the tube were vortexed for 30 seconds and exposed to ultrasound for 15 seconds. Plate counts were performed on the negative controls and the experimental disks using serial dilution and the pour plate technique using letheen agar. A percent bacterial reduction was determined for each disk by comparing the count for the

negative control disk to the experimental disks for each test organism.

In general, cidal activity increased for each disk as contact time increased. The 3% chlorhexidene disk showed the greatest cidal activity against all test organisms. It took a minimum of 60 minutes of contact time for the 2% and 3% disks to show large (>99.90%) bacterial reductions against all three test microorganisms. As the release of the antibiotic occurs by diffusion, this provides some indication of the efficacy of antibiotic release from the material.

Figure 8 shows the measured zones of inhibition around the test specimens. We have observed in related testing that the results obtained with zone inhibitions tests are asymptotic. That is, there is an antibiotic concentration beyond which the addition of more drug produces little effect. With CHX, this level is generally in the range of 2-5% drug loading by weight. On the basis of the bactericidal testing, we selected 3% as the level which was used in the in vivo tests of the dressing. As none of the experimental animals developed infection, those experiments did not demonstrate the efficacy of incorporating the antibiotic into the dressing.

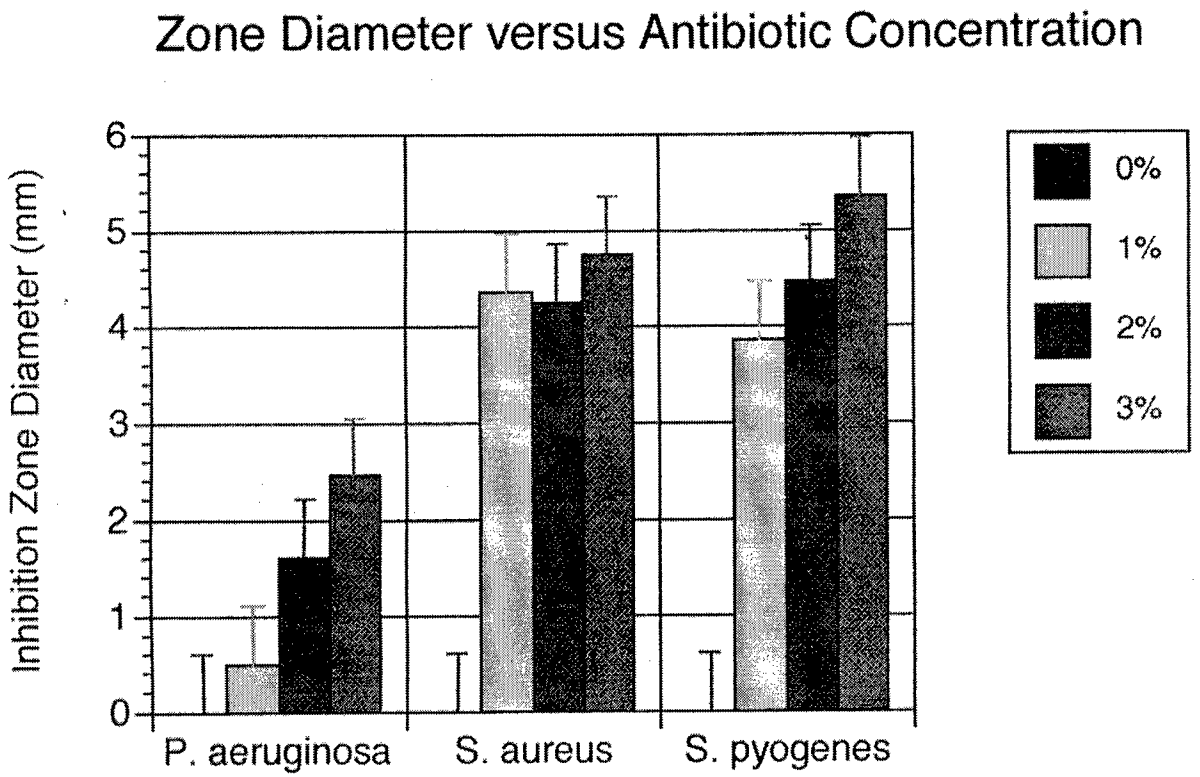

Figure 7. Zone inhibition for CHX with three microorganisms. In our testing of CHX loaded materials, concentrations in the range of 3-5% have yielded the best results. Further increases in the drug concentration produce little added benefit. A concentration of 3% was used in the external wound dressings.

Task V. Packaging, Shelf Life, and Toxicity Evaluations

Packaging and Shelf Life Studies

The material which was chosen to package the foam material and wound dressing is provided by Roll Print Packaging Products Incorporated, Addison IL. The material is RPP #26-1002 and is a heat seal coated composite of 0.5 mil oriented polyester, 1.0 mil aluminum foil, 2.0 mil polyethylene, and 5 pounds per ream of a primer coating from roll print. This composite is specifically designed to provide a metal appearance with strong peelable seals and nearly total barrier properties.

For field use, this metalized packaging seems particularly appropriate. It is also desirable for packaging the hydrophilic foam material, in particular. The foam is prepared with aromatic precursors, and it is UV sensitive. Opaque packaging is thus required. The loose hemostatic agent is packaged in 110 mg doses. Because of its low density (approximately 0.013 g/cm^3), this represents a volume likely to be sufficient for most purposes.

Because we have used commercially available thrombin in its manufacture, the formulation of the hemostatic agent includes benzelthonium chloride as a preservative. Other than this, we did not add any ingredient to stabilize the material. We studied the shelf life of the packaged hemostatic agent over a six month period.

Chromatography was carried out on a RSpak D18-613 column (6 mm x 150 mm) (Waters, 036576) with running back pressure about 500 psi (maximum 2842 psi). Acetonitrile/water/ammonium hydroxide (44/55/1) was applied as the mobile phase which is degassed and filtered before it was applied to the pump (Waters, Model 510). A flow rate of 0.89 cm/ml was maintained in the whole experiment, and the samples were introduced through a Waters U6K Universal LC Injector. The sample present was quantitatively analyzed by the means of a Waters Model 450 Variable Wavelength Detector, set at 0.4 AUFs with 254 nm as the wavelength, connected to a data process of Waters Data Module 746.

Samples of the hemostatic agent manufactured in August 1996 were compared with material which was produced in February 1997 for the toxicity tests. The older material had been kept refrigerated during storage.

Figure 8 shows chromatographs made using the above methodology for these materials. Comparing the chromatographs of the 6 months old and newly made material, we can conclude that fibrin monomer can maintain its stability over 6 months. While many protein based material are stored under refrigeration, we do not have a measure at this time of the shelf life of the hemostatic agent stored at room temperatures. Retrospectively, it would have been useful to conduct tests of material stored at room temperature for shorter periods of time to estimate the field life of the agent.

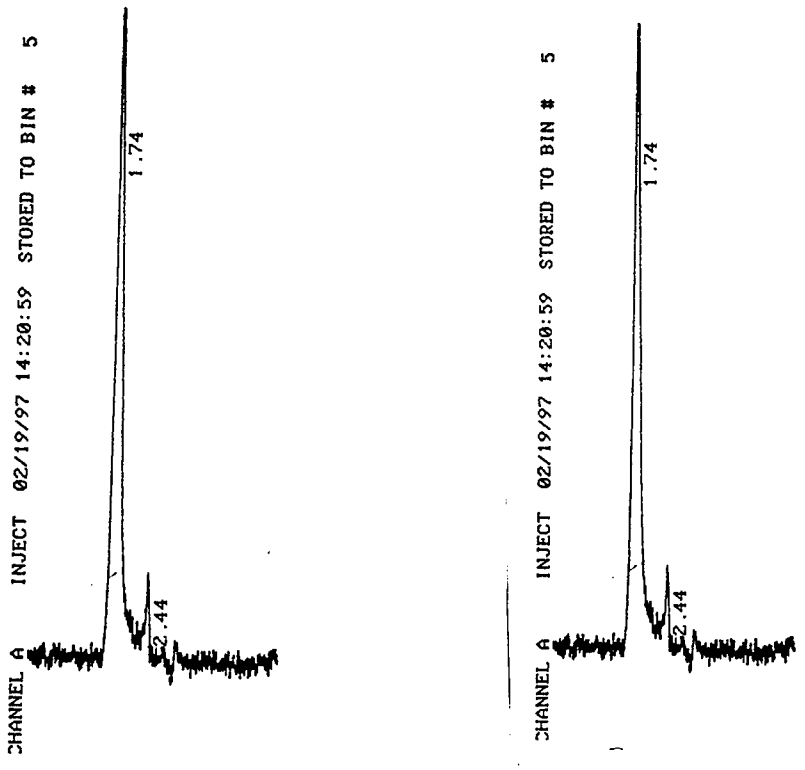

Figure 8. Chromatographs of samples of the hemostatic agent produced in August 1996 (left) and February 1997 (right). There has been no apparent change in the material over this 6 month duration.

Toxicity Testing

Toxicity testing of the foam delivery system was conducted in the first year of this program. These tests were conducted in the facilities of NAmSA (Norwood, OH), a commercial toxicological testing laboratory using standardized protocols. The foam samples were sterilized at WBI and supplied to NAmSA. Because of potential heat sensitivity of the foam material, we employ ethylene oxide gas sterilization for this purpose. The same lot of material was used for all of the tests which were conducted.

Toxicity tests of the hemostatic agent have also been completed. Those tests were performed by Toxicon Inc., Bedford, MA, a nearby toxicological test laboratory. Those tests were conducted in accordance with the FDA Good Laboratory Practices (GLP) regulations, 21 CFR, Part 58. The following ISO tests were performed: pyrogenicity, primary skin irritation, systemic injection, and complement activation.

These tests required the production of 10 g of the material, a volume of approximately 0.75 L. This amount represented a significant effort, since our pre-production set-up limits the amount of material which can be produced at a given time to less than 1.0 g. The material was supplied to Toxicon, and there it was compressed in sheets of known area to conduct the extractions required for these tests. Because the test protocols are based on

surface area, it is likely that evaluating the hemostatic agent in this way is an extremely severe test, since a low density powder, even when compressed, has significantly more surface area than solid samples of the same dimensions.

Results of Toxicity Testing

The foam delivery system passed all USP Class VI tests as described in the prior annual report of this program (April 1996). Those tests included: in vitro hemolysis, acute intramuscular injection, 30 day intramuscular injection, USP intracutaneous toxicity, USP systemic toxicity, Ames Mutagenicity, Kligman Maximization, and rabbit pyrogenicity. All test extracts met the USP requirements, and the foam material thus received Class VI certification from NAmSA.

The complete toxicity test results of the hemostatic agent itself are included in Appendix B. The material yielded negative results in the pyrogenicity, primary skin irritation, and systemic irritation tests. It did induce complement activation of C3 and C5 proteins in human plasma, most likely a consequence of the fact that it is made with proteins from a different species (bovine). By virtue of the delivery systems which have been developed for using the agent, however, the amount of material used and the resultant potential systemic exposure to this foreign material is minimized. Thus, we believe the hemostatic agent in its current form can be utilized without significant risk in human subjects.

The availability of recombinant proteins will ultimately eliminate even this as an issue. Recombinant human thrombin is currently available clinically, and fibrinogen is soon to follow. A formulation of the fibrin monomer based hemostatic agent made using recombinant proteins will thus be possible. While the current formulation virtually precludes viral disease transmission, the use of recombinant proteins will eliminate immunogenicity as a potential complication of its use.

Conclusions

A fibrin based hemostatic agent and wound dressing system specifically to be usable on the battlefield has been developed. The agent is a fibrin monomer based powder which is directly usable without any pre-mixing. In vivo tests indicate that: (1) the new hemostatic agent controls bleeding significantly faster than the only commercially available fibrin-based hemostatic agent, (Tisseel®, Immuno AG, Austria) when tested in a splenic injury model; (2) the tissue response and wound healing obtained with the new material and Tisseel® appear to be comparable; (3) the new material shows promise in the control of arterial bleeding when used on the surface of a hydrophilic sponge; and (4) the material yields a tissue tensile strength equivalent to surgical staples and with less localized inflammation at one week when it is used in an external, adhesive-backed wound dressing.

Because of its ease of use and effectiveness, this material has the potential for wide applicability in the treatment of trauma in civilian settings as well as its military uses.

Appendix A

In Vivo Test Results from the Cleveland Clinic Foundation

DAMD17-94-C-4039

"Tissue Adhesives for Battlefield Hemorrhagic Control"

Final Report for Subcontract

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Summary of Results

1. Chronic Tissue Healing Studies in Rabbit Splenic Injury Model

A. The mean bleeding times for the fibrin monomer (4.0 ± 2.9 min) and the fibrin monomer with aprotinin (5.7 ± 4.5 min) were significantly shorter than the mean bleeding time for Tisseel (24.2 ± 19.1 min).

B. At autopsy, there were no macroscopic signs of hemoperitoneum or severe inflammation.

C. All three preparations provided aufficient sealing of the wound without signs of secondary hemorrhage. These materials, however, elicited chronic inflammatory responses characterized by granuloma formation, when remaining in tissues.

2. Wound Dressing Studies for Deep Tissue Injuries, Rabbit Model

A. The mean bleeding times from femoral artery wounds were significantly less with application of hemostatic sponges which contained the fibrin monomer hemostatic agent (7.9 ± 3.3 min) than with application of hemostatic sponges without a hemostatic agent (15.9 ± 8.7 min).

3. Wound Dressing Studies for External Wounds in Pigs

A. All skin wounds treated with staples and those treated with the fibrin monomer patch were well healed at 1 and 4 weeks.

B. Mechanical testing showed that there were no significant differences in ultimate tensile strength of the wounds related to treatment except at four weeks in the skin of the abdomen.

C. Tissue healing occurred at the rate and with the strength comparable to that occurring with the skin staples. The advantage over the skin staples is that there is no acute inflammatory reaction. This appears inevitably with staples.

Task II. Chronic Tissue Healing Studies

Rabbit Splenic Injury Model

Materials

In chronic tissue healing studies, the basic fibrin monomer hemostatic agent (FM), the same fibrin monomer hemostatic agent containing aprotinin (FM+A), and one of the commercially available fibrin glues in Europe and Japan, Tisseel®, were tested to determine their effects on hemostasis and tissue adhesion using the controlled splenic trauma model in 36 New Zealand white rabbits.

Animals

A total of 36 male New Zealand white rabbits were used in this study. The mean body weight of these animals was 4.14 kg with an SD of 0.33 kg. All of the pre-and post-operative animal care was supervised by Dr. Munoz-Ramirez, Staff Veterinarian. All animal procedures were performed in compliance with the regulations stated in the Guide for the Care and Use of Laboratory Animals by the U.S. Department of Health and Human Services (NIH publication No. 86-23, revised in 1985). All procedures were approved by the Animal Research Committee of The Cleveland Clinic Foundation.

Surgical Procedures

The animals were anesthetized with I.M.ketamine (35 mg/kg) and xylazine (5 mg/kg). Intravenous injections of ketamine (7 mg/kg) and xylazine (1 mglkg) were added for maintenance of the anesthesia.

After adequate anesthesia was achieved, the left flank was shaved. The animal was placed in the supine position, and the left flank was prepped with betadine.

A left subcostal oblique incision, 5 cm long, was made. The external oblique , internal oblique , and transversus muscle were dissected, and the abdomen was entered. The spleen was exposed, and its size recorded.

Three parallel scalpel wounds, 2 mm deep and 7 mm long, were made on the anterolateral splenic surface using a specially- designed incision length and depth guide (Figure 1). One hemostatic agent was applied to each wound; the #1, #2, and #3 wounds were treated by FM, FM+A, and Tisseel®, respectively (Figure 2). Gentle compression was applied with a gauze sponge for the first three minutes, then the wounds were observed. Subsequently, the wounds were checked every

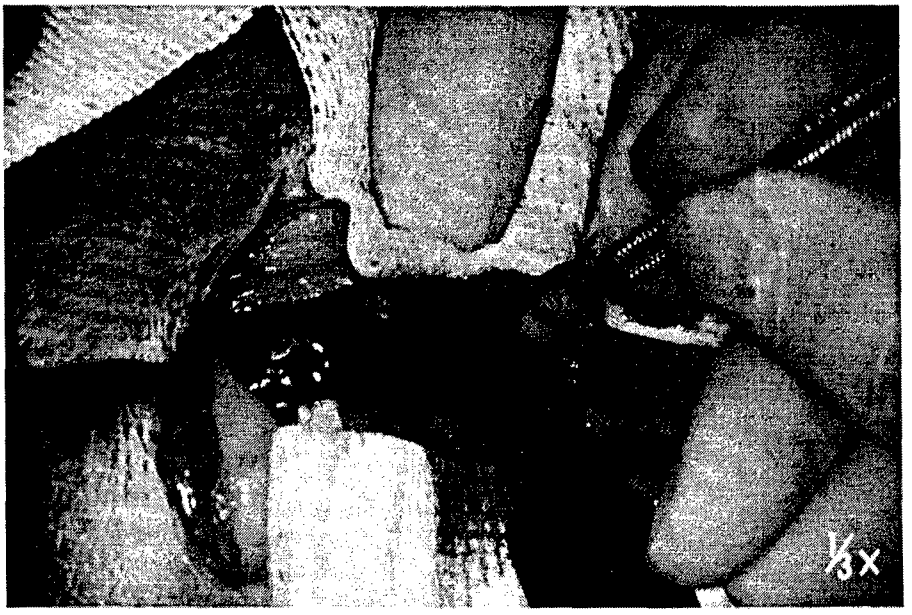


Figure 1. Three parallel scalpel wounds, 2 mm deep and 7 mm long, were made on the anterolateral surface of the spleen using a specially designed incision length and depth guide.

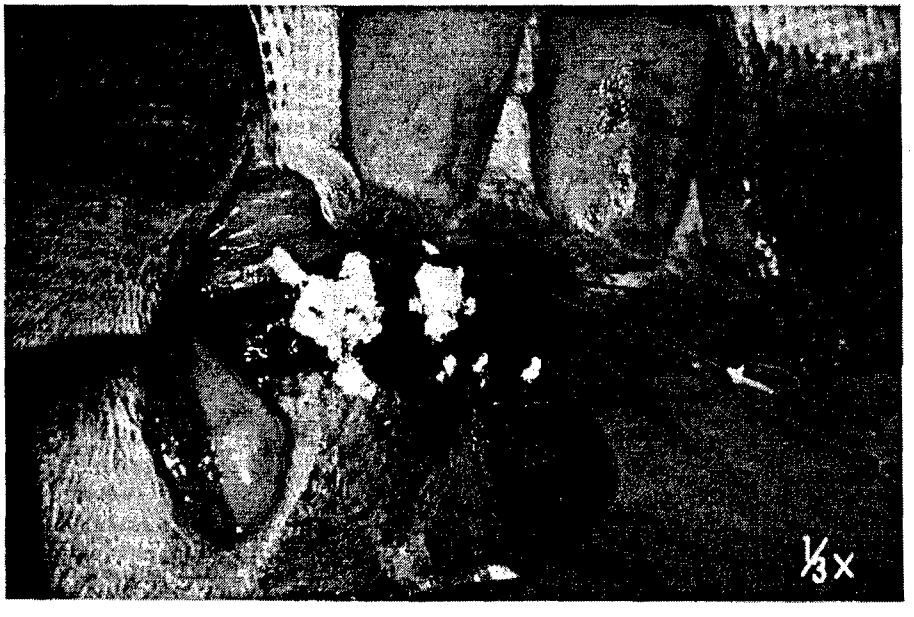


Figure 2. One hemostatic agent was applied to each wound; the #1(far left wound in the figure), #2 (center), and #3(right) wounds were treated by FM, FM+A, and Tisseel, respectively. Both FM and FM+A are formulated in a powder form, while Tisseel is liquid and is applied by a syringe and needle. In four randomly selected cases, a fourth wound was added as a non-treated control.

Table 1

Fibrin Based Hemostatic Agent
Splenic Injury Model in Rabbit
Bleeding Time (min)

Exp#	#1(FM)	#2(FM+A)	#3(Tisseel)	#4(Gauze)
96009	3	7	15	
96010	3	13	26	
96011	5	3	60	60<
96012	3	3	70	70<
96013	3	6	13	
96014	5	4	15	
96025	3	3	16	
96026	4	22	58	
96028	3	5	14	
96029	6	4	10	
96037	3	4	32	
96038	4	10	8	
96039	3	4	15	
96043	3	3	13	
96044	5	5	10	
96045	4	4	13	
96049	3	3	7	
96050	7	10	11	
96051	3	3	10	
96052	3	3	12	
96062	3	3	30	
96063	4	4	68	
96064	3	6	46	
96068	4	5	10	
96069	4	4	15	
96070	3	6	48	
96073	3	3	9	
96074	3	6	10	
96075	3	3	10	
96076	3	3	56	
96077	3	3	15	
96078	20	10	29	60<
96084	3	3	8	
96085	3	4	15	
96086	3	20	50	
96087	3	3	23	60<
Ave.	4.00	5.69	24.17	
SD	2.91	4.50	19.21	

#1(FM) : the basic fibrin monomer hemostatic agent
#2(FM+A) : the basic fibrin monomer hemostatic agent containing aprotinin

minute by gently removing the gauze sponge. The time that was required for each wound to obtain complete hemostasis was recorded as the bleeding time. In four animals, the #4 wound, 2 mm deep and 7 mm long, was made for the measurement of the bleeding time treated with conventional sponges without hemostatic agents.

Results of the bleeding times

Table 1 shows the bleeding times in each experiment. The mean bleeding times for FM, FM+A, and Tisseel® were 4.0 ± 2.9 min, 5.7 ± 4.5 min, and 24.2 ± 19.1 min, respectively. Both FM and FM+A had significantly shorter bleeding times than Tisseel® ($p<.001$) (Figure 3). ANOVA was used for this statistical analysis. Tisseel® was easily removed from the wound surface when the gauze sponge was elevated for inspection. In contrast, FM and FM+A adhered well to the wound edge and surface, as judged by the continued presence of whitish fibrin clots around the wound. The wounds treated with conventional sponges without hemostatic agents continued to bleed for more than 60 minutes, and hemostatic agents were applied in order to stop the bleeding.

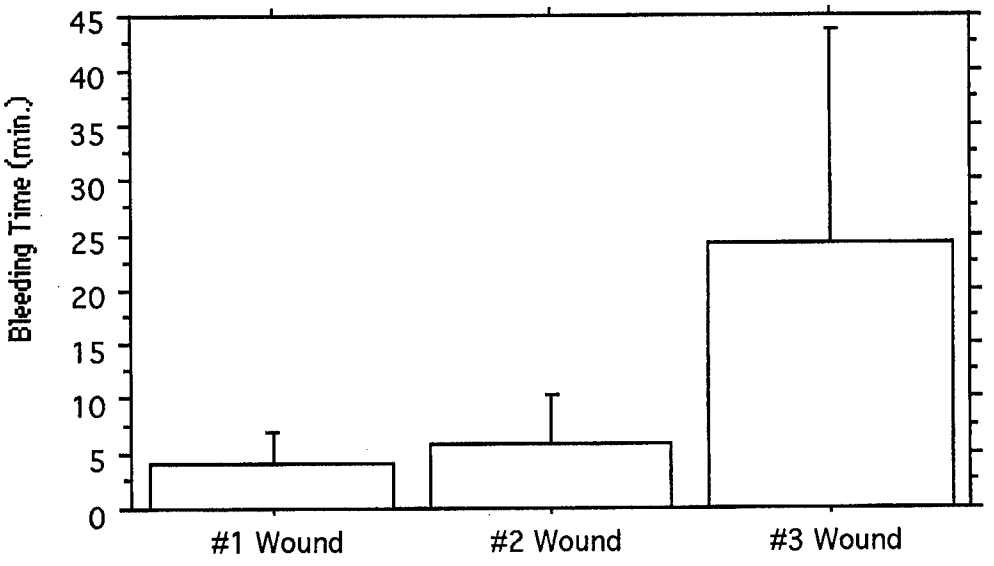


Figure 3. The mean bleeding time and S.D. for FM (#1 wound), FM+A (#2 wound), and Tisseel. (#3 wound) was 4.0 ± 2.9 min, 5.7± 4.5 min, and 24.2 ±19.1 min, respectively. Both FM and FM+A had significantly shorter bleeding times than Tisseel. ($p<.001$).

Postoperative course

The animals were observed for signs of internal bleeding in the immediate postoperative period. All recovered uneventfully. No sign of hemoperitoneum or recurrent bleeding was observed postoperatively. Prophylactic antibiotics, a mix-

ture of benzathine-procaine penicillin (40,000 U/kg), were injected intramuscularly for 2 days. Food and water were provided ad libitum after recovery from anesthesia. There were no postoperative deaths. Two animals bit their sutures producing dehiscence of the abdominal skin wound. Betadine solution was applied to the wounds of these twice a day until autopsy.

Autopsy / Macroscopic findings

All animals were sacrificed after 2 days (n=12), 1 week (n=12), or l month (n=12) to observe the trauma sites macroscopically and to obtain specimens for microscopy. The abdominal wounds healed well except in two animals (Exp.#96010 and Exp.#96028) of 36. These two, as described, bit the sutures of their wounds, producing small skin dehiscences without discharge. The muscle layers in those two animals were free from discoloration, discharge or dehiscence.

No hemoperitoneum was observed at autopsy in any of the 36 animals. In 14 of 36, mild adhesions were noted between the peritoneum of the abdominal wall at the site of laparotomy and the omentum or the visceral organs. In 32 of 36, the spleen was mildly adhered to the surrounding tissues, mainly to the omentum and/or small intestine, which were easily detached (Figure 4, 5). No hematoma or sizable granuloma suggestive of preceding hematoma formation was noted.

The average and standard deviation values of the physical data of the spleens were 2.03 ± 0.72 g in weight, 5.9 ± 1.0 cm in length, 1.1 ± 0.2 cm in width and 0.5 ± 0.1 cm in thickness.

In the 2 day experiments, residual FM and FM+A were noted in all of the # l and #2 wounds, mainly as white membranous substances, while the residual Tisseel® was recognized macroscopically in the #3 wounds in l0 out of 12 cases.

In the 1 week experiments, all of the #1 and #2 wounds were covered by an ivory- yellow substance; 11 of 12 #3 wounds were covered by similar substances.

In the l month experiments, eleven of the 12 # l wounds, all of the #2 wounds, and l1 out of the 12 #3 wounds were covered mainly by such ivory- yellow substances. It was difficult to determine the exact locations of the wounds macroscopically, especially in the l month experiments.

In conclusion, no signs of hemoperitoneum or severe inflammation were observed. The materials appeared to be able to achieve hemostasis in splenic injuries without severe inflammation or adhesion in rabbits.

Histologic findings

The histology specimens were fixed with an alcohol-based, recently available fixative, HistoChoice, embedded either in paraffin or in plastics

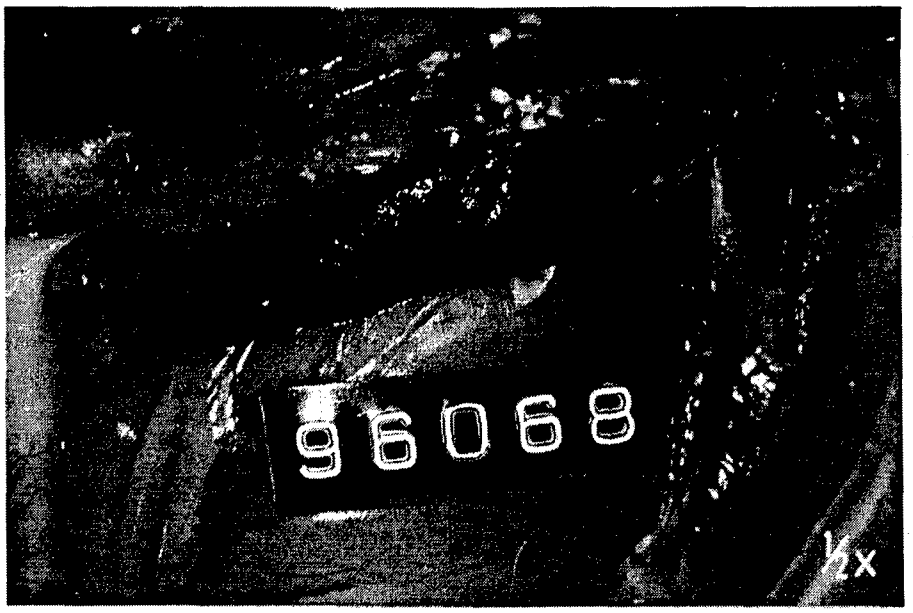


Figure 4. The figure illustrates a typical appearance of the wounded sites of the spleen after two days. The peritoneum is clean without any sign of secondary hemorrhage or generalized inflammation. Thin fibrinous adhesion between the spleen and the peritoneum was easily dissected bluntly.

Figure 5. The peritoneal cavity after one month of procedure appears to be unremarkable without signs of secondary bleeding, hematoma or infection. The spleen is partially adherent to the reitoneum, omentum and adjacent intestines with a thin fibrous tissue. The size of the spleen did not show any **appreciable** change.

(2-hydroxyethylmethacrylate, HEMA and glycomethacrylate, GMA), sectioned, and stained with hematoxylin-eosin (H&E) and Masson's trichrome.

The cross-sections obtained from the l week-experiments showed the presence of a thin layer of eosinophilic, homogeneous FM covering the splenic serosal surface and the wound providing adequate sealing without signs of secondary hemorrhage (Figure 6). The triangular-shaped wound was filled with fresh blood clot with a mild cellular response along the wound edge. Collagenization in the wound edge was minimal (Figure 7). Around the FM a moderate degree of inflammatory cell response was noted. These inflammatory cells were composed of both polymorphonuclear (PMN) and mononuclear (MN) leukocytes which were migrating through the underlying splenic capsule. Foreign body giant cells (FBGC) were rarely seen. The amount of FM on the splenic capsule and in the wound differed significantly from one case to another, and hence, so did the extent of inflammatory cell response. With FM+A and Tisseel, the wound also appeared well-sealed and the qualitative histologic features were similar to those seen with FM. A quantitative assessment of the cross-sectional area of the wounds, the amount of tissue adhesives remaining in and on the wound areas, the degree of inflammatory responses and collagenization, and the time course and comparisons of these parameters among three preparations were performed using a computer-aided morphometric system. The results are reported below. One week after surgery, blood clots in the wound were nearly absorbed and were replaced by a loosely collagenized tissue with negligible amounts of inflammatory response, when there was no hemostatic agent remaining in the wound area (Figure 8). When the hemostatic agent was present, there was a layer of chronic inflammatory cells along the wound edge with frequent FBGCs. Collagenization in this inflammatory cell layer was clearly observed at this time (Figure 9). By one month the wound had healed with a small amount of fibrous tissue without recognizable inflammatory cell infiltrates. When adhesives remained, they elicited granuloma formation around the degrading materials. The granuloma was composed of degraded inflammatory cells, epithelioid cells, MNs, tissue capsule, and FBGCs. Figure 10 shows such granuloma formed on the splenic surface surrounding degrading FM+A one month after application. Figure 11 is a section cut parallel to the wound treated with Tisseel showing a granulomatous response along the material lying outside of the splenic capsule. In this case it is noted that the inflammatory response expands beyond the splenic capsule causing significant accumulation of epithelioid cells and macrophages in the splenic parenchyma, especially in the subcapsular region.

All three hemostatic agents appear to have provided sufficient hemostasis without secondary hemorrhage. The addition of aprotinin for stabilization of fibrin does not seem to have produced histologic features supporting its intended use. Although the amount of FM, FM+A, and Tisseel remaining in the wounds was not

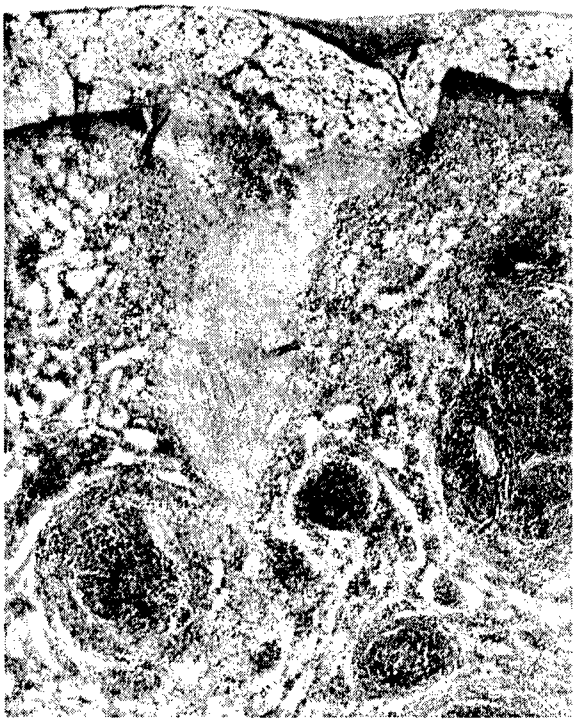


Figure 6. A cross section of the spleen with a two-days old scalpel wound treated with FM. The wound is triangular in shape and filled with exudate with a mild cell infiltration along the wound edge. No exudation of blood is observed into the splenic parenchyma from the wound edge. The wound opening and the adjacent splenic capsule is covered by a layer of polymerized fibrin, approximately 2505M in thickness, which appears to be tightly adherent to the capsular surface (Histology specimen No. 96013-1, magnification: 268X).

Figure 7. With a higher magnification, after 2 days, the wound space is shown to be filled with a loose fibrin meshwork with a small number of nucleated cells. The wound edge is well demarkated without insudation of blood components into the adjacent parenchyma. Cellular accumulation along the wound edge is also mild. In this case, no FM is seen in the wound (Histology specimen No. 96014-1, magnification: 1014X).

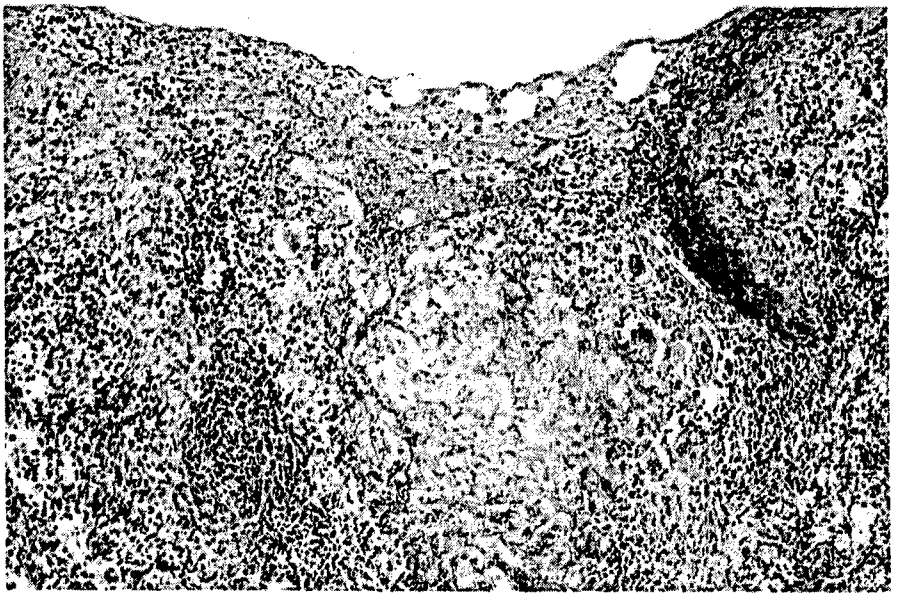


Figure 8. A histology section from 1 week experiment shows a cluster of FM in the center of the wound surrounded by a layer of inflammatory cells and foreign body type giant cells. The polymerized FM appears as lightly stained, homogeneous complex strand-like structures with a small amount of invading inflammatory cells. The outer layer of this inflammatory cell layer is sightly collagenized. The tissue bridging the disrupted splenic capsule, a part of which appears as a dense fibrous tissue curling down along the wound edge in the right upper portion of the figure, is also collagenized (Histology specimen No. 96009-1, magnification: 1014X).

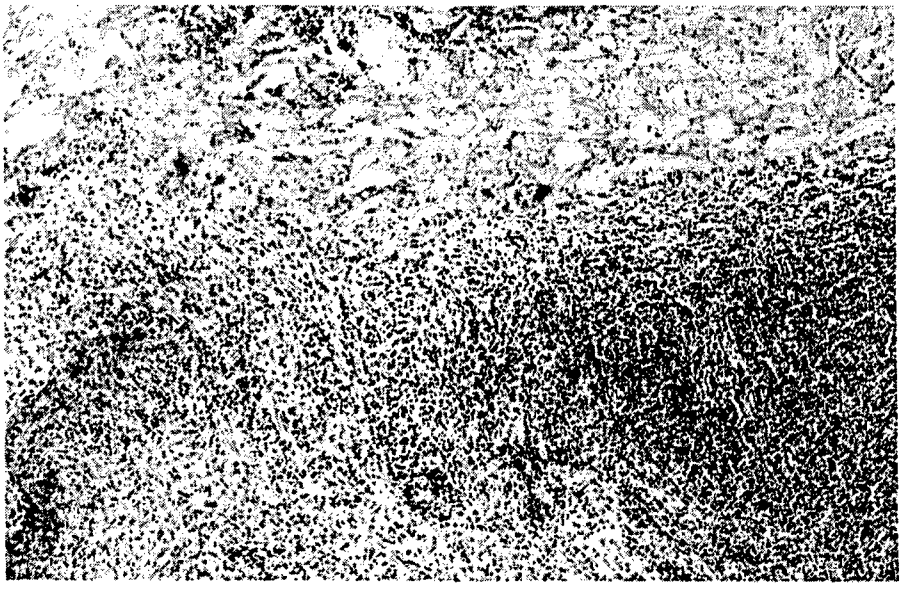


Figure 9. A higher magnification view of the FM+A and wound edge of the spleen for 1 week. The upper portion of the figure contain the cross sections of lightly stained, homogeneous polymerized FM+A which is surrounded by a layer of moderately intense inflammatory cells. The spaces between the strands of FM+A are filled with cell fragments and degradation products. A large cluster of nucleated cells in the right middle portion of the figure is the white pulp of the spleen, and is the normal structural components of the spleen. The cut edge of the splenic capsule is seen in the left lower corner of the figure (Specimen No. 96039-2, magnification: 1014X).

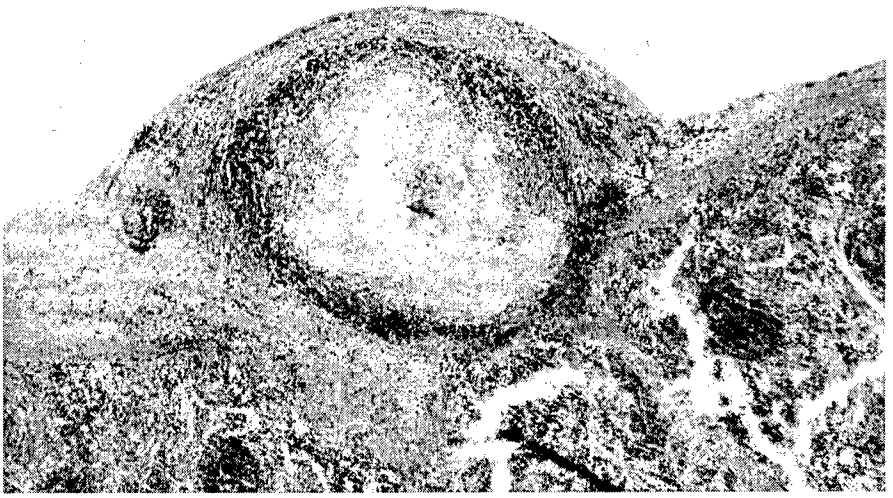


Figure 10. A granuloma found one month after in the wound site of the spleen, which was treated with FM+A. The granuloma is formed surrounding a hydrolyzing polymerized FM+A, and has a typical cellular composition including epithelioid cells, chronic inflammatory cells, and a fibrous tissue capsule in an order of inside to outside. Foreign body type giant cells are frequently observed with a higher magnification in the outer portion of the inflammatory cell layer (Histology specimen No. 96025-2, magnification: 504X).

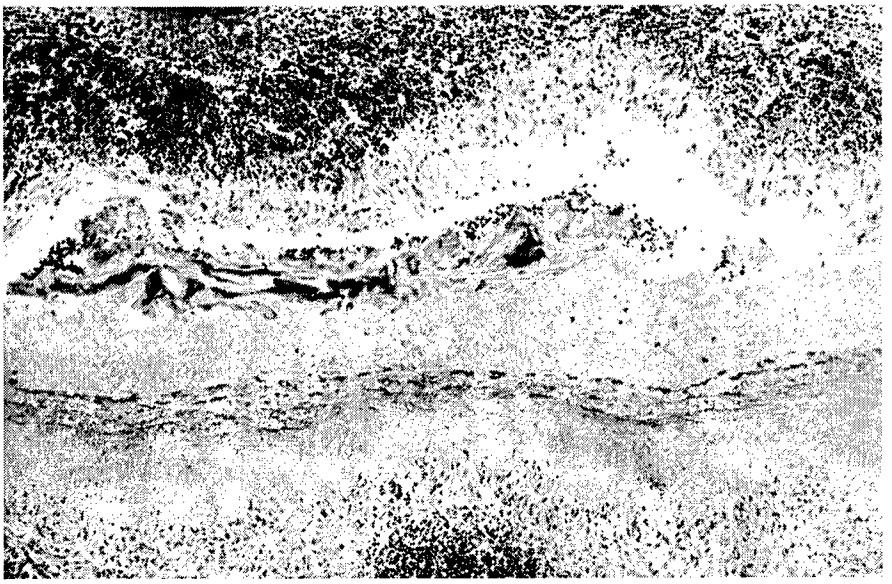


Figure 11. A cross section of the splenic capsule cut parallel to the wound treated with Tisseel. After one month, Tisseel is still remaining on the surface of the fibrous tissue capsule of the spleen, which is shown as multiple fiber structures running from the left to the right of the mid-portion of the figure. Above this capsule there is polymerized, but partially degrading Tisseel, surrounded by a thick layer of large epithelioid cells with a lightly stained cytoplasm. This epithelioid cell layer reaches below the fibrous capsule of the spleen extending into the parenchyma of the spleen, approximately 100-1505M in depth. Toward the top of the figure, a dense chronic inflammatory cell layer is noted (Histology specimen No. 96025-3, magnification: 1014X).

controlled, the persistent presence of hemostatic agents appears to delay the wound healing process with a foreign body type reaction and significant granuloma formation. Since the origins of fibrin monomer, thrombin, and fibrinogen used in preparing the three materials are xenogenic to our current rabbit animal model, the inflammatory response elicited by these preparations may have been exaggerated due to potential immunologic reactions.

Quantification Of The Inflammatory Response To Tissue Adhesives

Using a computerized image analysis system (Bioquant Meg IV Morphometric System, R&M Biometrics, Nashville, TN) several key features revealed in trichrome-stained histology sections were quantified. These features included: (l) discontinuous distance of the serosal membrane of the spleen, (2) depth of wound (3) area of the residual amount of adhesive remaining in the wound, (4) area of inflammation (leukocyte infiltration), and (5) area of collagenization. Measured values were statistically analyzed to determine variations among the three types of adhesives with respect to inflammation, collagenization, and rate of adhesive adsorption. This analysis, however, has limited usefulness in this experiment because the amount of hemostatic agent which was applied to each wound was not controlled. A method of clarifying this data was explored by determining if the amount of residual adhesive correlated with either the area of the inflammatory layer or with the amount of collagenization for a specific adhesive type and time point. No correlations were observed for comparisons and the data seems inconclusive. Despite these results , this type of analysis could prove to be useful when a protein from one species is placed into another species. With a specifically-designed study, such a quantification method could provide information on the resorption rate of the adhesives and the degree of inflammation and collagenization that may be associated with different materials.

Task III. Delivery System Development and Testing

Rabbit Femoral Injury Model

In the wound dressing studies for deep tissue injuries, the hemostatic sponge including the fibrin- based hemostatic agents and the same hemostatic sponge without hemostatic agents were tested in rabbits using a controlled femoral artery trauma model. The rabbits, which were used in the splenic injury study, were reused just before sacrifice on the day of autopsy.

Eight rabbits were used to establish the methods to produce a well- controlled injury of the femoral artery, which was approximately 1 - 2 mm in diameter. At

first, 19 - 21G needle injuries were made, covered by a sponge, then checked by gently removing the sponge. This method, however, seemed unsuitable for testing the materials because the hemostatic agents and thrombi were removed by the removal of sponges to check the wounds. It was decided that bleeding from the wound should be checked without removing the sponges. When the wounds made by 19G needles were treated by the test sponges and checked without removing the sponges, hemostasis of the wounds was achieved within 3 - 4 minutes. More damaging, but well controlled injuries needed to be considered. Thus, transsection of the femoral artery was performed. To control the size of this wound, we decided that regions of the left and right superficial femoral arteries with the same diameters, 1 mm, should be transsected. After transsection, the hemostatic sponge with the hemostatic agents was applied to the left femoral artery, while the same sponge without hemostatic agents was applied to the right femoral artery in the 8 rabbits. Gentle compression was applied for the first three minutes; then the wounds were checked by releasing pressure but with the sponges left in place. Subsequently, the wounds were checked every minute without removing the sponges. The time that was required for each wound to obtain complete hemostasis was recorded as the bleeding time (Table 2). The mean bleeding times for the hemostatic sponge with the hemostatic agents and the same sponge without hemostatic agents were 7.9 ± 3.3 min and 15.9 ± 8.7 min, respectively. The hemostatic sponge with the hemostatic agents had a **significantly** shorter bleeding time than the same sponge without hemostatic agents ($p = 0.028$).

Table 23

Hemostatic Sponge with Fibrin Based Hemostatic Agents
Femoral Artery Injury Model in Rabbit
Bleeding Time (min)

Exp.#	Hemostatic Sponge	Control (without hemostatic agents)
96074	5	8
96076	7	23
96077	5	6
96078	4	20
96084	9	26
96086	8	8
96085	13	26
96087	12	10
Average	7.88	15.88
SD	3.31	8.69

Studies of External Wound Dressings in Pigs

Materials

In studies of the dressings for use in external wounds, the test dressing with the fibrin-based hemostatic agents and surgical skin staples (Reflex® One 8535, Richard-Allan Medical, Richard, MI), as a control, were tested using a controlled skin injury model in pigs. The test dressing was an elastic, semi-occlusive dressing which incorporated hemostatic agents and antibiotics in a layer as a delivery system for the fibrin based hemostatic agents. In this study, chlorohexidine was incorporated into the release layer as an antibiotic.

Animals

A total of 12 female pigs, 22.64 ± 3.87 kg in weight, were used. All pre- and post-operative care was supervised by Dr. Munoz-Ramirez, Staff Veterinarian. All procedures were performed in compliance with regulations stated in the Guide for the Care and Use of Laboratory Animals by the U.S. Department of Health and Human Services (NIH publication No. 86-23, revised in 1985). All procedures were approved by the Animal Research Committee of The Cleveland Clinic Foundation.

Surgical procedures

The animal was anesthetized with ketamine (20 mg/kg, I.M.) and thiopental (15 mg/kg, I.V.), intubated and maintained on respirators with 30% oxygen in air and 1-2% halothane.

After adequate anesthesia was achieved, the abdomen and the back were clipped and shaved and prepped in a sterile manner with betadine scrubbing and subsequently painted with betadine solution.

The locations for the incisions were marked. The skin blood flow around each wound was measured by a laser doppler flowmeter (Model ALF 21 D, Transonic SystemS Inc. Ithaca, NY) before and after making the incisions, and after the treatments were applied. To make these measurements the flow probe was placed on the skin near the center of each wound on both sides of the incision and within 5 mm of the incision.

Full thickness skin incisions, 2 cm long, were made bilaterally on the abdominal and dorsal regions with a#15 scalpel. The incisions were made parallel to the skin lines, cranial to caudal on the abdomen and medial to lateral on the back. The wounds were made in this manner because it was felt that the test dressing could not maintain close skin approximation if the tension against approximation was

high. Wounds which are parallel to the skin lines receive such mild tension against the approximation that the test dressing seemed to be able to maintain good skin approximation. The left sided wounds were treated with the test dressings with the hemostatic agents (Figure 12). The right sided wounds were closed with skin staples (Figure 13).

Postoperative course

All of the animals recovered uneventfully from anesthesia and surgery. Systemic prophylactic antibiotics were not given. Food and water were provided ad libitum after recovery from anesthesia. The skin wounds were protected from contamination. The trunk of the animal was wrapped using a Self-Adherent Wrap (Coban™, 3M, St. Paul, MN) for at least 3 days. If the test dressing was inadvertently removed by the animal, a new test dressing was immediately re-applied to the wound until POD 7. All skin staples and test dressings were removed on POD 7.

Autopsy / Macroscopic Findings

All animals were electively sacrificed 1 week after surgery (n = 6) or 4 weeks after surgery (n = 6) to observe the trauma sites and to obtain specimens for microscopic examination. The skin blood flow around each wound was measured before sacrifice.

In the 1 week group, all of the wounds that were treated by the test dressings were healing well without discoloration, discharge or dehiscence (Figure 14). Although all of the wounds treated by surgical staples were also well-healed without discharge or dehiscence, six out of 6 abdominal and 2 out of 6 back wounds treated by surgical staples were accompanied by small pink swellings, which appeared to be associated with staple perforations (Figure 15). Skin specimens were taken from the wounds for wound tear strength tests and histologic analyses.

In the 4 week group, all of the wounds, both control and test treatment, were healed so well that it was difficult to determine their locations (Figure 16, 17). Skin specimens were taken from the wounds for wound tear strength tests and histologic analyses.

In conclusion, the dressings tested and surgical staples appeared macroscopically to be comparable in participating in the healing process when there was low tension against approximation of the wound edges.

Mechanical Testing

Specimens for tensile testing were collected at the time of autopsy using the foil

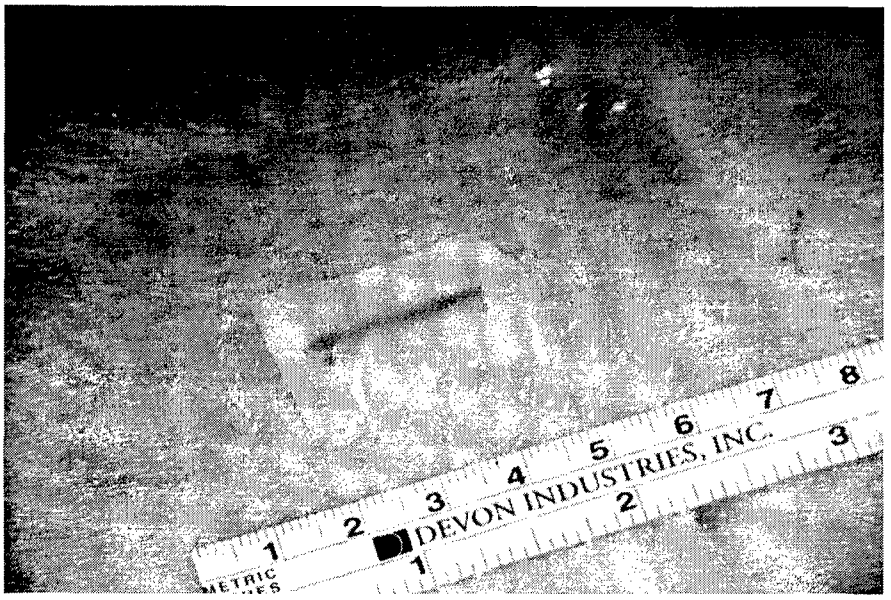


Figure 12. A 2 cm long, sharp, scalpel wound was placed in the full thickness of the skin in both abdominal and dorsal regions of a pig. The wound was then covered by the test dressing material, which has a semi-opaque, porous area in the center for the delivery of the hemostatic agent. The picture shows an adequate approximation of the wound by this dressing without bleeding.

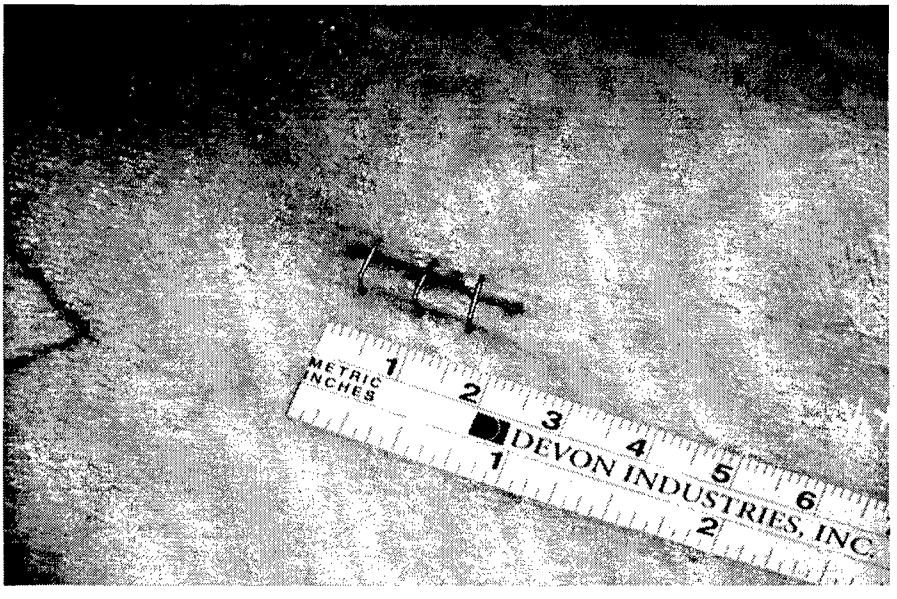


Figure 13. The contralateral wound was approximated by three skin staples as a control.

Figure 14. After one week the left abdominal wound treated by the test dressing appears as a thin line with slight redness along the wound edge, which measures 19 mm long and 0.5 mm wide. Approximation of the wound is sufficient without sign of dehiscence, infection or hemorrhage/discharge (Exp.# 97002).

Figure 15. An appearance of the right abdominal wound approximated by staples after one week (20 mm long, less than 0.5 mm wide). The wound and the holes caused by stapling are covered by dark brownish scabs with a typical centipede pattern inherent to staples and sutures. Although approximation of the wound edge is sufficient and the wound is dry, swelling and redness of the skin are always observed along the wound especially in the areas around the holes of the staples (Exp.# 97002).

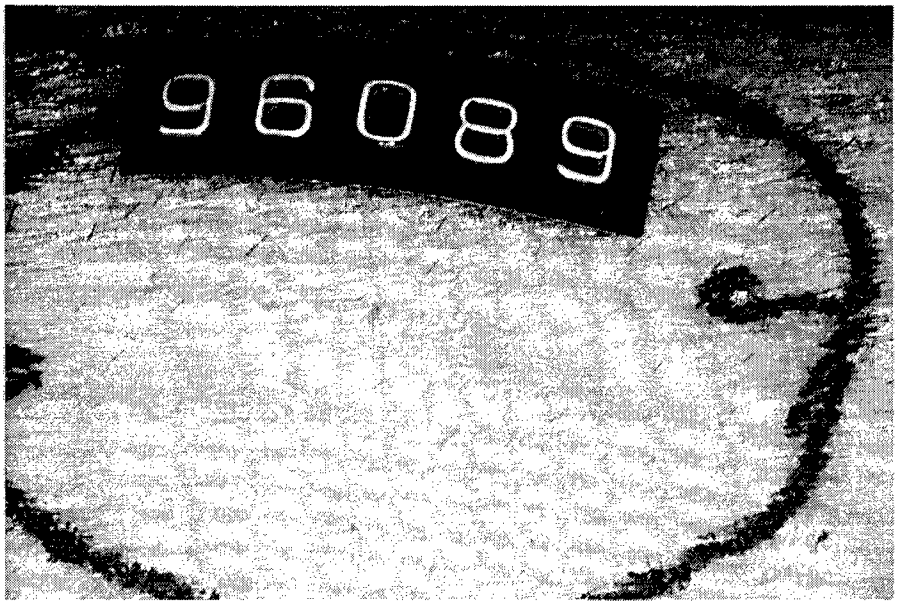


Figure 16. The left abdominal wound treated by the test dressing healed completely after 4 weeks, and measures 17 mm in length, although it is hardly recognizable. No appreciable scar formation, discoloration or skin indentation was noted (Exp.# 96089).

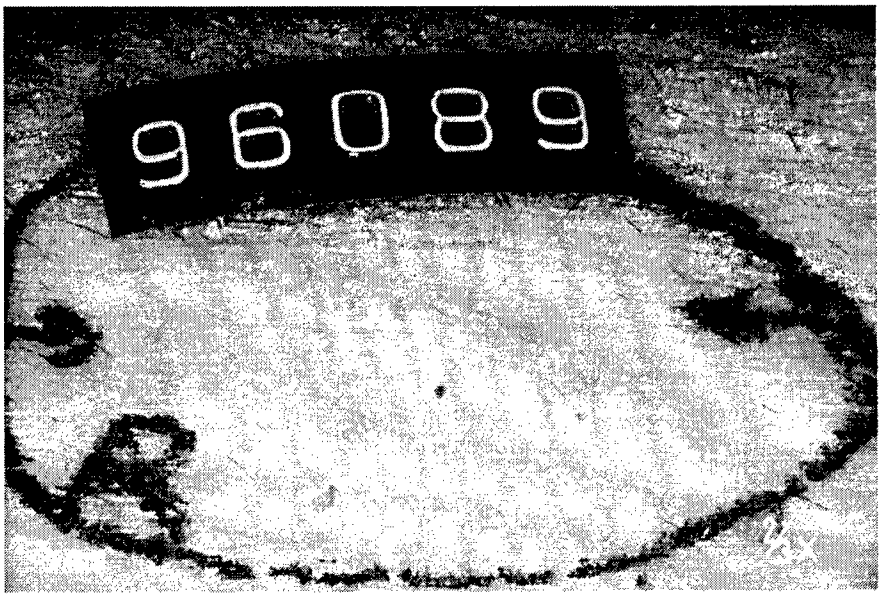


Figure 17. The counterpart wound treated by staples is also clean and healed well. By this time inflammation around the staple holes and the centipede pattern are gone and the wound edge is hardly recognizable (Exp.# 96089, 4 weeks).

method. Each of the four cicatrices or scars was identified, and after macroscopic descriptions were taken, each cicatrix was traced with a skin marker, and the tensile test specimen shape was drawn around it (Figure 18). All of the scars were 1.8 to 2.0 cm long. The specimens for tensile testing, which were all 1 cm wide at the wound, were taken from the center of the scar leaving 4 to 5 mm on each side for histologic specimens. For the first three cases (Exp.#'s 96089, 96090, and 96091) rectangular specimens, 1.0 cm wide by 15 cm long, were taken. These specimens provided a 1.0 cm wide area for clamping into the tensile testing machine, and some of the specimens slipped in the grips. In the remaining 9 cases, the specimen shape was changed to a dog bone configuration to reduce slippage. This configuration is shown in Figure 19. To cut the 1.0 cm wide region, a 1.0 cm wide guide of ductile copper was bent to the shape of the pig and then placed transversely across the center of the scar. Then, two parallel razor blades, which were held 1.0 cm apart by a spacer, were placed over the guide and then pulled along it to cut the skin. These two tools are shown in Figure 20. The tapered and grip portions of the dog bone were traced from a template and cut by scalpel. After the cuts were made around the specimens, they were dissected from the underlying tissues. When a specimen was free, its thickness in an area near the wound was measured to the nearest 0.1 mm with a micrometer. The specimen was then placed on a towel which was moistened with lactated Ringer's solution. When each pig was finished, its four specimens were transported to the lab for tensile testing.

Tensile testing was performed on an MTS Model 952 universal tensile testing machine using a 22.7 kg (50 lb.) load cell. The specimens were removed from their moist environment, quickly clamped into the tensile tester, and pulled to failure at a rate of 50 mm/sec. A digital acquisition system recorded instantaneous force and length and the peak force at failure. The failed specimens were returned to the moist environment. They were subsequently observed to determine where the failure had occurred and to observe the appearances of the failed surfaces. This latter information was used to determine if the wound location had been properly identified and included in the test strip. (Proper identification was difficult in some of the four week specimens, and this method was used to eliminate some data points from the final results.) The peak force values were divided by the cross-sectional area (1.0 cm width x measured thickness) to yield ultimate tensile stress.

Based on the observation of the failed specimens, one 1-week and four 4-week specimens were not included in the final results. The indications for this elimination included failure at the grips or misidentification of the wound such that it was not included in the specimen. Wound failure was characterized by edges which were perpendicular to the long axis of the test specimen and straight, and the newly exposed surfaces were smooth. In specimens which did not include the

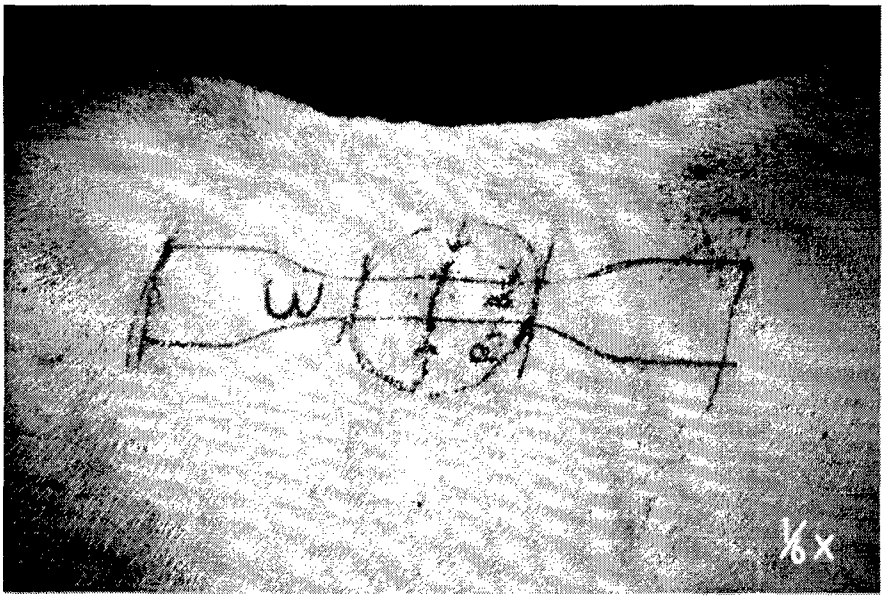


Figure 18. The picture illustrates the shape and the size of the skin specimen obtained for mechanical testing at the time of autopsy.

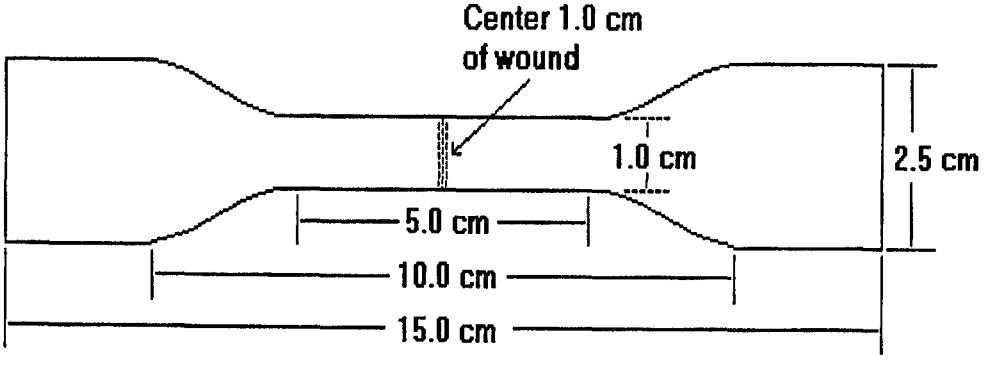


Figure 19. A schematic drawing of the dimensions of the skin flap prepared for the mechanical testing.

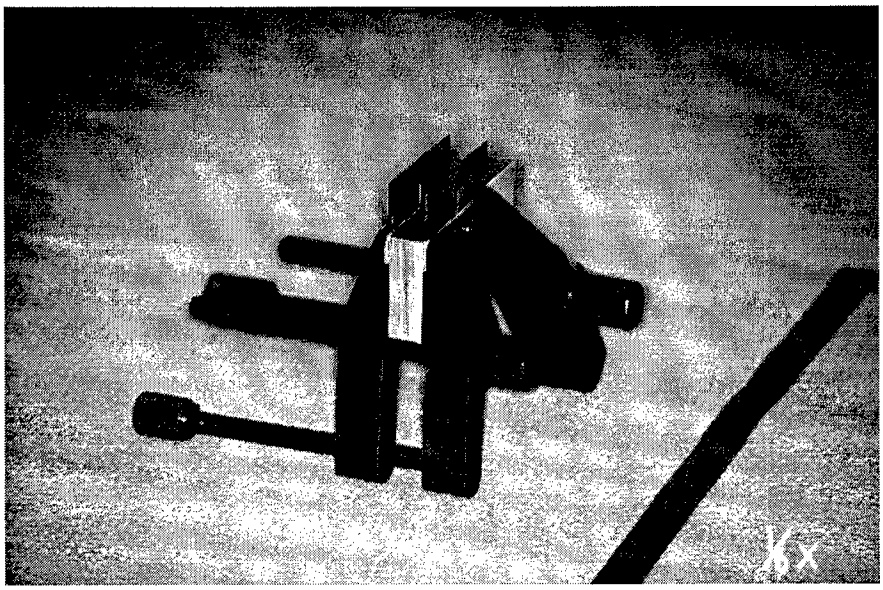


Figure 20. A copper guide(right) and a specially designed razor blade holder for cutting 1.0 cm wide portions of tensile testing specimens.

wound, the failure lines appeared torn with rough edges, and they were perpendicular to or diagonal to the long axis. Some specimens were checked by histology to verify if the failure had occurred at the wound or not.

The results for the l week group are shown in Table 3 for the abdomen and in Table 4 for the back. The results for the 4 week group are shown in Table 5 for the abdomen and in Table 6 for the back. Statistical analyses of the data were performed by ANOVA and then by paired t-tests.

At 1 week there were no differences in ultimate tensile stress between the stapled wounds and the fibrin monomer wounds for either the abdomen or the back. At 4 weeks, the stapled wounds on the abdomen were significantly stronger than the fibrin monomer wounds on the abdomen ($P = 0.0044$). There was no difference between the back wounds at 4 weeks.

Histologic findings

The skin incisions approximated by surgical staplers and the test dressings showed a continuous epidermal layer closing the wound at one week; the presence of significant indentation and invagination of the epidermis was a frequent finding. No signs of dehiscence, wound infection or secondary internal bleeding were noted in either treatment.

A significant difference observed in stapled and test dressing treatments was, as suggested by the macroscopic findings, multiple loci of acute inflammatory cell accumulations, hemorrhage and edema apparently associated with the staples in

Table 3

Ultimate Tensile Stress for Abdomen Wounds, 1 Week

Right - Control, Staples			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
97001	1.6E-05	2.34	0.146
97002	1.4E-05	3.20	0.229
97003	1.3E-05	1.88	0.145
97005	1.2E-05	14.26	1.188
97006	2.2E-05	4.97	0.226
97007	1.5E-05	6.15	0.410
		Average	**0.391**
		Standard Deviation	**0.403**
Left - Fibrin Monomer Patch			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
97001	0.7E-05	1.24	0.177
97002	1.2E-05	2.57	0.214
97003	0.9E-05	1.41	0.157
97005	1.0E-05	4.61	0.461
97006	1.4E-05	3.28	0.234
97007	1.0E-05	2.98	0.298
		Average	**0.257**
		Standard Deviation	**0.111**

Table 4

Ultimate Tensile Stress for Back Wounds, 1 Week

Right - Control, Staples			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
97002	2.5E-05	2.42	0.097
97003	2.4E-05	1.59	0.066
97005	2.2E-05	5.64	0.256
97006	2.7E-05	5.37	0.199
97007	2.4E-05	5.86	0.244
		Average	**0.172**
		Standard Deviation	**0.086**
Left - Fibrin Monomer Patch			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
97001	3.1E-05	6.36	0.205
97002	2.7E-05	4.84	0.179
97003	2.6E-05	4.21	0.162
97005	2.1E-05	13.47	0.641
97006	2.7E-05	11.54	0.427
97007	2.5E-05	6.05	0.242
		Average	**0.310**
		Standard Deviation	**0.189**

Table 5

Ultimate Tensile Stress for Abdomen Wounds, 4 Weeks

Right - Control, Staples			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
96089	1.2E-05	66.35	5.529
96091	1.9E-05	70.10	3.689
96100	1.2E-05	59.52	4.960
96102	1.0E-05	48.46	4.846
		Average	**4.756**
		Standard Deviation	**0.771**
Left - Fibrin Monomer Patch			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
96089	1.1E-05	14.25	1.295
96091	1.8E-05	16.56	0.920
96100	1.2E-05	23.84	1.987
96102	0.9E-05	21.94	2.438
		Average	**1.660**
		Standard Deviation	**0.681**

Table 6

Ultimate Tensile Stress for Back Wounds, 4 Weeks

Right - Control, Staples			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
96089	3.3E-05	106.25	3.220
96090	3.0E-05	98.63	3.288
96091	2.9E-05	145.36	5.012
96100	2.0E-05	93.80	4.690
96101	2.9E-05	173.03	5.967
96102	2.1E-05	138.18	6.580
		Average	**4.793**
		Standard Deviation	**1.369**
Left - Fibrin Monomer Patch			
Experiment #	Area (m^2)	Maximum Load (N)	Stress (MPa)
96089	2.8E-05	88.11	3.147
96090	3.1E-05	109.31	3.526
96091	2.8E-05	124.79	4.457
96100	1.9E-05	54.62	2.875
96101	2.9E-05	104.29	3.596
96102	2.3E-05	148.96	6.477
		Average	**4.013**
		Standard Deviation	**1.321**

the animals closed with that method (Figure 21). In contrast, the inflammatory response was negligible with the test dressings (Figures 22, 23). The Stratum corneum, Stratum, granulosum, and Stratum germinativum are visible. In the dermis there is a distinct separation which is filled with fibrinous exudation with a mild inflammatory cell infiltrate (Figures 21, 23). In the abdominal skin, this separation seems to be wider in the deeper layer of the dermis treated with the test dressing, resulting, in conjunction with the indentation of the epidermal layer, mentioned above, in thinning of wound edge of the dermal connective tissue layer (Figure 22). This rather poor apposition of the wound edge in the dermis was not observed in the skin of the back. This was thought to be best explained by the fact that the abdominal skin is considerably thinner and more friable than the skin of the back and that pigs usually rub their abdomens against the ground or the wall of the pen eliciting continuous distorting forces to the wounds which disturb healing. By one month, the dermis was approximated with well-collagenized fibrous tissue with minimal amounts of inflammatory cells. Figure 24 shows a cross- section of the skin incision of the back approximated by staples at one month. Indentation of epidermis was not significant. The dermis was connected with a newly formed fibrous tissue, approximately 50-100 micrometers wide. Focal inflammation sites seen in one week specimens were not observed at one month. The dermis did not show any sign of thinning. The histologic features of the skin incision in the back treated with the test dressing showed healing without persistent inflammatory responses or thinning of the dermis (Figure 25). FM was not observed in either one week or one month specimens.

The thinning of the dermal layer seen in the abdominal skin incisions treated with the test dressing was thought to explain a weaker mechanical strength of the wound at the one month period compared with the abdominal wounds approximated by staples, which provided better approximation of the wounds when subjected to the external forces. No differences were noted in dermal thickness in the back skin incision groups, regardless of treatment.

Skin Blood Flow

The skin blood flow in the vicinity of each wound was measured with a laser doppler flowmeter before and after making the incisions, and after the treatments were applied. It was also measured just before sacrifice on the day of autopsy. For this measurement, the flow probe was placed on the skin near the center of each wound on both sides of the incision and within 5 mm of the incision.

Table 7 shows the skin blood flow data. In the case of the wound treated by the test dressing, it was not possible to accurately measure the flow after the dressing was in place. The pre-values were approximately 4 ±6 ml/min/100g. These values appeared to be maintained throughout the study.

Figure 21. A photomicrograph of the cross section of the abdominal skin wound one week after approximation with skin staples. The epidermal layer is continuous with indentation and invagination corresponding to the location of the original cut wound. The formation of the stratum corneum, stratum granulosum and stratum germinativum is recognized. In the deeper layer, however, the dermis is not yet healed and the wound is still filled with proteinaceous exudation with mild cellular response. Clusters of inflammatory cells are visible in the subepidermal layer along the entire length of the specimen (Histology specimen No. 97001-1, magnification: 504X).

Figure 22. A cross section of the skin wound treated with the test dressing for one week. The epidermis is healed and is continuous, but with indentation and invagination. The deeper layer of the dermis is separated with resultant upward migration of the subcutaneous fatty tissue. This upward migration and the indentation of the epidermis lend the wound edge of the dermis available for subsequent fibrous healing much smaller with the resultant thinning of the healed wound. This separation of the dermis is obvious in the abdominal wound location, but far less recognizable in the dorsal wound location. (Histology specimen No. 97002-2, magnification: 504X).

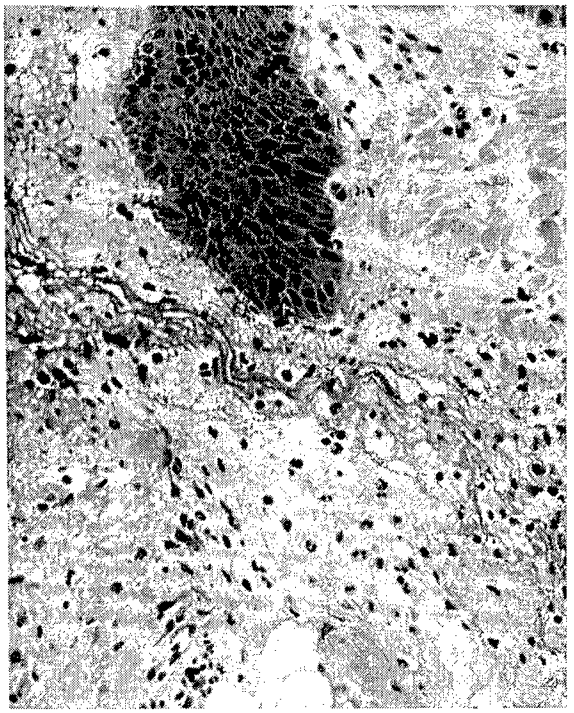


Figure 23. A higher magnification view of the wound in the dermis shown in Figure 22. The presence of two different types of cells are noted in the epidermis protruding into the wound in the dermis (right hand side of the picture). The space (1.5 mm wide) between two separated dermal layers (top and bottom portions of the figure) is filled with loose fibrinous materials with a small number of inflammatory cells. In the left side the subcutaneous fatty tissue is visible. The distance between the epidermis and this fatty tissue is very narrow and less than 1 mm. (Histology specimen No. 97002-2, magnification: 2,023X).

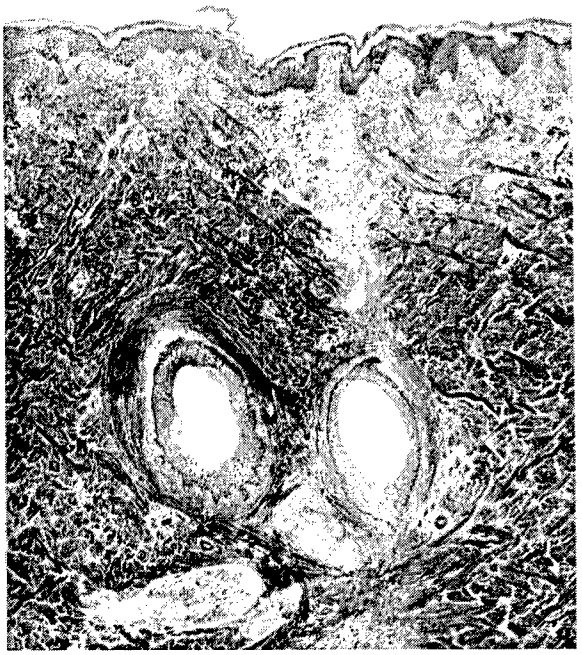


Figure 24. The dorsal skin wound approximated with the test dressing material, after one month. The epidermis is continuous without significant indentation or thickening. The wound edge in the dermis is connected with a thin band of matured collagenized tissue without sign of inflammation. In the dorsal location no appreciable thinning of the dermis is noted (Histology specimen No. 96092-3, magnification: 504X).

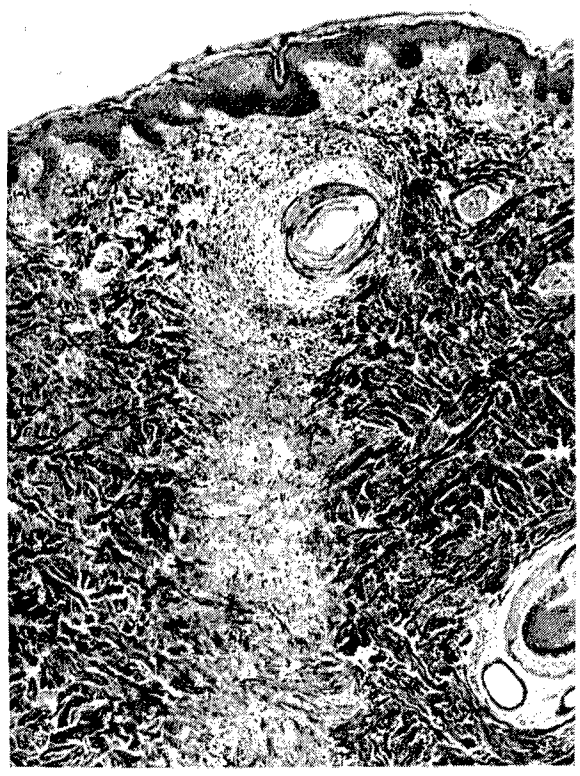


Figure 25. The cross section of the dorsal skin wound approximated with staples, after one month. The dermis covering the wound is healed well and shows only a very localized, small indentation. The healing of the dermis is comparable to that seen in Figure 24. There is no inflammatory response or thinning of the dermis (Histology specimen No. 96102-4, magnification: 504X).

Table 7

Test dressing with Fibrin Based Hemostatic Agents
Skin Injury Model in Pig

Skin Blood Flow (ml/min/100g)

		Abdomen test dressing	Abdomen staples	Back test dressing	Back staples
pre-incision	mean	6.73	4.35	4.84	5.55
	SD	3.28	2.47	1.97	3.39
post-incision	mean	5.42	4.51	4.34	4.55
	SD	3.07	2.65	2.08	2.00
post-treatment	mean	2.86	4.09	3.17	5.23
	SD	2.06	1.91	1.20	2.58
1 week	mean	4.92	5.25	3.51	3.29
	SD	2.05	1.21	0.59	1.05
4 weeks	mean	5.23	5.24	5.01	5.39
	SD	1.61	1.33	2.23	2.79

Appendix B

Toxicity Tests of the Fibrin Monomer Based Hemostatic Agent/Tissue Adhesive

TOXIKON FINAL REPORT: 97G-0346

RABBIT PYROGEN TEST (Material Mediated) - ISO

Author
Ahmad N. Ali, Ph.D.

Final Report Date
March 31, 1997

MANAGEMENT OF THE STUDY

Performing Laboratory
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor
Whalen Biomedical
11 Miller Street
Somerville, MA 02143

TABLE OF CONTENTS

STUDY SUMMARY

The test article, Hemostatic Agent, was evaluated for its potential to produce a pyrogenic response when tested in albino rabbits. Based upon the criteria of the protocol, the test article meets the requirements of the Pyrogen Test and is therefore considered non-pyrogenic.

QUALITY ASSURANCE STATEMENT

Based on a review of this study report, protocol and Standard Operating Procedures utilized, and inspection of study events listed below, this test conformed to the FDA Good Laboratory Practice (GLP) regulations, 21 CFR, Part 58.

The Sponsor was responsible for all test article purity, stability, and characterization data as specified in 21 CFR, Parts 58.105 and 58.113. The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED MANAGEMENT	DATE REPORTED STUDY DIRECTOR
DOSING	03/12/97	03/12/97	03/12/97
RAW DATA	03/31/97	03/31/97	03/31/97
FINAL REPORT	03/31/97	03/31/97	03/31/97

Shari L. Sheroke, B.S.
Quality Assurance Officer

3/31/97
Date

STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

This study meets the technical requirements of the protocol. The study also meets with the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58.

Study Director: Ahmad N. Ali, Ph.D.

Company: Toxikon Corporation

Signature: 

Date: 03/31/97

Study Supervisor: Shirley Orfao, B.S.

VERIFICATION DATES:

The study dates were as follows:

Protocol Effective Date:	02/05/97
Test Article Receipt:	02/25/97
Project Log Date:	02/25/97
Stock Training/Sham Test:	03/08/97 - 03/11/97
Technical Initiation:	03/12/97
Technical Completion:	03/12/97
Final Report Date:	03/31/97

1.0 PURPOSE

The pyrogen test was designed to determine the presence of chemical pyrogens in extracts of solid materials in order to limit to an acceptable level the risks of febrile reaction in the patient to the administration of the product concerned. The test involved measuring the rise in temperature of albino rabbits following the intravenous injection of a test article. It is designed from products that can be tolerated by the test rabbit in a dose not to exceed 10 ml per kg, within a period of not more than 10 minutes.

2.0 REFERENCES

The test was based on the following references:

2.1 Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity, ISO/ANSI/AAMI 10993-11:1993.

2.2 Biological Evaluation of Medical Devices - Part 12: Sample Preparation and Reference Materials, ISO/CD (Draft 1993) 10993-12.

3.0 COMPLIANCE

The study conformed to all applicable laws and regulations. Specific regulatory requirements included the current FDA 21 CFR, Part 58 - Good Laboratory Practice for Nonclinical Laboratory Studies; AAALAC, "Guide for the Care and Use of Laboratory Animals," National Research Council, 1996. (NIH) (OPRR), "Public Health Service Policy on Humane Care and Use of Laboratory Animals," Health Research Extension Act of 1985 (Public Law 99-158 November 20, 1985), Reprinted 1996; USDA, Department of Agriculture, Animal and Plant Health Inspection Service, 9 CFR Ch.1 (1/1/95 edition), Subchapter A-Animal Welfare, ISO 10993-2 (1993).

4.0 IDENTIFICATION OF TEST AND CONTROL ARTICLES

The following information was supplied by the Sponsor wherever applicable; it did not apply to confidential information. The Sponsor was responsible for all test article characterization data as specified in the GLP regulations. Test and control articles (exclusive of extracts) that are mixed with carriers require verification of concentration, homogeneity, and stability. Returning of the samples of test and control article mixtures to the Sponsor for characterization was not applicable.

Test Article Name: Hemostatic Agent
CAS/Code #: Not Supplied by Sponsor (N/S)

Lot/Batch #: 9702
Physical State: Powder
Color: White
Stability: Refrigerate
Solubility: N/S
Storage Conditions: Room Temperature
Safety Precautions: Standard Toxikon Laboratory Safety Precautions

4.2 Negative Control Article (Toxikon Supplied)
Control Article Name: USP 0.9% Sodium Chloride for Injection (NaCl)
QC Inventory #: CSC-96-10-005-VIV
Physical State: Liquid
Color: Clear
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions Apply

5.0 IDENTIFICATION OF TEST SYSTEM

5.1 Animals Used in the Test

Number and Species: 4 New Zealand white rabbits (*Oryctolagus cuniculus*)

Sex: 1 male and 3 females

Weight/Age range: 1.70 and 3.50 kilograms / 10 to 12 weeks old (adult)
Weighed to the nearest 10 gm

Animal Purchase: Eastern Rabbit Breeding Laboratory, Taunton, MA

Animal Selection: selected from a larger pool of animals and examined to ensure lack of adverse clinical signs.

Animal Identification: tattoo

Acclimatization: minimum 5 days under the same conditions as the actual study.

5.2 Animal Care and Maintenance

Housing: individually housed

Cages: suspended stainless steel

Bedding: hardwood chips

Animal room temperature: 68±5°F

Animal room relative humidity: 30-70%

Air exchanges per hour: 10 to 15

Lights: 12-hour light/dark cycle, full spectrum fluorescent lights

The laboratory and animal rooms are maintained as limited-access facilities.

Animal rations: Agway Prolab Rabbit Formula, *ad libitum*
Water: tap water, *ad libitum*

There were no known contaminants present in the feed, water, or bedding expected to interfere with the test data.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

6.1 Albino rabbits were used in this study because they historically have been used in pyrogen evaluation studies and the guidelines have no alternative (non-animal) methods. The animal species, number and route were recommended by the ISO 10993-11 guidelines.

6.2 The test article was administered through a solution compatible with the test system. This was the optimal route of administration available in this test system.

7.0 EXPERIMENTAL DESIGN

7.1 Prior to using the rabbits in the pyrogen test, they were conditioned not more than 7 days before use in a Sham Test. This included all the steps as directed in the pyrogen test, except that the material injected intravenously was USP 0.9% Sodium Chloride Solution for Injection and the temperature readings were taken at 1, 2, and 3 hours subsequent to injection.

7.2 Test and control articles were prepared as described in Section 8.0. All apparatus was prepared according to the requirements of ISO 10993-11.

7.3 The control article was 0.9% Sodium Chloride for Injection USP, treated in the same manner as the test article.

7.4 Single-use disposable syringes and hypodermic needles were used for the administration of the test article. Glassware used in testing was rendered free from pyrogens by heating at 200°C for not less than 3 hours.

7.5 A Digital Thermometer (Yellow Springs Instruments Co., Inc. Yellow Springs, OH) with thermistor probes (accuracy of 0.1°C) was used to measure rectal temperatures. A probe was inserted into the rectum of the test rabbit to a depth of not less than 7.5 cm. After a period of temperature stabilization (approximately 1 minute), the body temperature was taken.

7.6 The test was performed in the Pyrogen Test Room, designated solely for the pyrogen testing, under environmental conditions similar to those under which rabbits are housed and free from disturbances which may excite them. The rabbits were restrained for the course of the study and are not offered food or water during the three hour test period. Since rectal temperature measuring probes are inserted throughout the testing period, the rabbits were restrained with light-fitting neck stocks that allowed the animals to assume a natural resting position.

7.7 Not more than 30 minutes prior to injection of the test article, the baseline temperatures were determined. This served as a baseline for determining any increase in temperature resulting from the injection of the test article. None of the test animals exhibited a baseline temperature which varied by more than 1°C from each other or which was greater than 39.8°C.

7.8 Body temperatures were recorded at 30 minute intervals between 1 and 3 hours subsequent to injection.

7.9 At the end of the observation period, the animals were returned to the general colony.

8.0 DOSAGE

8.1 Preparation of Test Article
The test and control articles were prepared in NaCl at ratios specified by ISO 10993-12.

The test article was extracted at a ratio of 120 cm^2 in 20 mL of 0.9% USP NaCl at 37$\pm$2°C for 24 hours.

Properly prepared test article was placed in an extraction bottle and NaCl was added. The extraction medium completely covered the test article.

The extracting medium (control article) was prepared for parallel treatments and comparisons. The control article was prepared in the same manner as the test article.

Each extract was agitated vigorously prior to administration.

8.2 The test or control article was injected into the marginal ear vein of each rabbit at a dose based on the body weight of each animal. Test article extracts were injected at a dose not to exceed 10 ml per kg or as specified by Sponsor. Each injection was completed within 10 minutes after the start of administration.

9.0 EVALUATION CRITERIA

9.1 Temperature decreases are considered as zero rise.

9.2 If no rabbit shows an individual rise in temperature of 0.5°C or more above the baseline temperature, the test article meets the requirements for the absence of pyrogens.

9.3 If any rabbit shows an individual temperature rise of 0.5°C or more, a retest should be conducted using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.5°C or more, and if the sum of the eight individual maximum temperature rises does not exceed 3.3°C, the test article meets the ISO requirements for the absence of pyrogens.

10.0 RESULTS (Table I)

The temperature increases for the treated animals were 0.2, 0.2, and 0.0°C. The increases did not exceed the test limit for the maximum individual temperature rise. The temperature increase of the control animal was 0.1°C.

11.0 CONCLUSION

The test article, Hemostatic Agent, was evaluated for its potential to produce a pyrogenic response when tested in albino rabbits. Based upon the criteria of the protocol, the test article meets the requirements of the Pyrogen Test and is therefore considered non-pyrogenic.

12.0 RECORDS

12.1 Original raw data is archived at Toxikon Corporation.

12.2 A copy of the final report and any report amendments is archived at Toxikon Corporation.

12.3 The original final report and a copy of any protocol amendments or deviations is forwarded to the Sponsor.

12.4 All unused test article shall be discarded by Toxikon, per Sponsor's request.

12.5 Final reports shall not be reproduced except in full, without the written authorization/approval from Toxikon.

13.0 CONFIDENTIALITY AGREEMENT

Statements of confidentiality were as agreed upon prior to study initiation.

14.0 POLICY ON PAIN AND SUFFERING IN ANIMALS

No evidence of pain and suffering were observed in the animals.

15.0 ANIMAL USAGE

The Sponsor assures that, to the best of their knowledge, this study did not unnecessarily duplicate previous testing.

TABLE I

PYROGEN TEST DATA

Test Article: Hemostatic Agent

Technical Initiation: 03/12/97 Lot #: 9702

Species: New Zealand White rabbit Dose: 10 ml/kg

Test Parameters	Animals			
	70332 (Treated)	**70333** (Treated)	**70334** (Treated)	**70328** (Control)
Weight (kg)	2.09	2.43	1.98	2.13
Dose (ml)	20.9	24.3	19.8	21.3
Probe #	7	8	9	10
Baseline Temp (°C)	39.4	39.2	39.4	38.9
1 Hour Temp	39.6	39.3	39.0	38.9
1.5 Hour Temp	39.3	39.4	39.2	39.0
2.0 Hour Temp	39.2	39.3	39.2	39.0
2.5 Hour Temp	39.2	39.3	39.1	38.9
3.0 Hour Temp	39.2	39.4	39.1	39.0
Temp Increase*	0.2	0.2	0.0	0.1

* Temperature increases based upon maximum temperatures recorded at 1, 2, and 3-h observations.

TOXIKON FINAL REPORT: 97G-0350

PRIMARY SKIN IRRITATION - ISO

Author
Ahmad N. Ali, Ph.D.

Final Report Date
March 31, 1996

MANAGEMENT OF THE STUDY

Performing Laboratory
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor
Whalen Biomedical
11 Miller Street
Somerville, MA 02113

TABLE OF CONTENTS

STUDY SUMMARY

The test article, Hemostatic Agent (Sodium Chloride and Cottonseed Oil Extracts), was evaluated for its potential to produce Primary Skin Irritation after a single topical 4 hour application to the skin of albino rabbits. The test article is considered a negligible irritant.

QUALITY ASSURANCE STATEMENT

Based on a review of this study report, protocol and Standard Operating Procedures utilized, and inspection of study events listed below, this test conformed to the FDA Good Laboratory Practice (GLP) regulations, 21 CFR, Part 58.

The Sponsor was responsible for all test article purity, stability, and characterization data as specified in 21 CFR, Parts 58.105 and 58.113. The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED MANAGEMENT	DATE REPORTED STUDY DIRECTOR
CLINICAL OBSERVATIONS	03/14/97	03/14/97	03/14/97
RAW DATA	03/31/97	03/31/97	03/31/97
FINAL REPORT	03/31/97	03/31/97	03/31/97

Shari L. Sheroke

Shari L. Sheroke, B.S.
Quality Assurance Officer

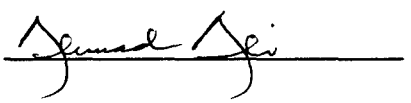
3/31/97
Date

STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

This study meets the technical requirements of the protocol. The study also meets with the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58.

Study Director: Ahmad N. Ali, Ph.D.

Company: Toxikon Corporation

Signature: 

Date: 03/31/97

Study Supervisor: Laurence Lister, B.S.

VERIFICATION DATES:

The study dates were as follows:

Protocol Effective Date:	02/14/97
Test Article Receipt:	02/25/97
Project Log Date:	02/25/97
Technical Initiation:	03/12/97
Technical Completion:	03/15/97
Final Report Date:	03/31/97

1.0 PURPOSE

The purpose of the test is to evaluate the potential of the test article to produce primary dermal irritation after a single topical exposure to the skin of albino rabbits.

2.0 REFERENCES

The test was conducted based upon the following tests:

2.1 Biological Evaluation of Medical Devices - Part 10: Tests for Irritation and Sensitization, ANSI/AAMI/ISO 10993-10:1995.

2.2 Extraction procedures were based upon the standard titled Biological Evaluation of Medical Devices - Part 12: Sample Preparation and Reference Materials, ISO/CD (Draft 1993) 10993-12.

2.3 Draize, J.H. "Dermal Toxicity", Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics, Association of Food and Drug Officials of the U.S., Topeka, Kansas, 1965, pp 46-59.

3.0 COMPLIANCE

The study conformed to all applicable laws and regulations. Specific regulatory requirements included the current FDA, 21 CFR, Part 58 - Good Laboratory Practice for Nonclinical Laboratory Studies; AAALAC, "Guide for the Care and Use of Laboratory Animals," National Research Council, 1996. (NIH) (OPRR), "Public Health Service Policy on Humane Care and Use of Laboratory Animals," Health Research Extension Act of 1985 (Public Law 99-158 November 20, 1985), Reprinted 1996; USDA, Department of Agriculture, Animal and Plant Health Inspection Service, 9 CFR Ch.1 (1/1/95) edition, Subchapter A-Animal Welfare. ISO 10993-2, 1992.

4.0 IDENTIFICATION OF THE TEST AND CONTROL ARTICLES

The following information was supplied by the Sponsor wherever applicable; it did not apply to confidential information. The Sponsor was responsible for all test substance characterization data as specified in the GLP regulations. Test and control articles (exclusive of extracts) that are mixed with carriers require verification of concentration, homogeneity, and stability. There were no samples of test and control article mixtures to be returned to the Sponsor for characterization.

4.1 Test Article Name: Hemostatic Agent
CAS/Code Number: Not Supplied by Sponsor (N/S)
Lot/Batch Number: 9702

Physical State: Powder
Color: White
pH: Not Supplied by Sponsor (N/S)
Density: N/S
Stability: Refrigerate
Solubility: N/S
Expiration Date: N/S
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

4.2 Negative Control Articles (Toxikon Supplied)

4.2.1 Control Article Name: USP 0.9% Sodium Chloride Injection (NaCl)
QC Inventory #: CSC-96-10-005-VIV
Physical State: Liquid
Color: Clear
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions Apply

4.2.2 Control Article Name: Cottonseed Oil (CSO)
QC Inventory #: CSC-96-11-005-VIV
Physical State: Liquid
Color: Yellow
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions Apply

5.0 IDENTIFICATION OF THE TEST SYSTEM

5.1 Animals Used in the Test:

Number and species: 6 New Zealand Albino Rabbits (*Oryctolagus cuniculus*)

Sex: 3 males and 3 females

Weight/Age range: not less than 2 kg / 10 to 12 weeks old (adult)
Weighed to the nearest 10 gm

Animal identification: ear tattoo

Health status: healthy, unused in other experimental procedures

Animal source: registered commercial breeder; Eastern Rabbit Breeding Labs.
Taunton, MA

Acclimatization: minimum of 5 days under the same conditions as for the actual test.

Animal selection: selected from a larger pool of animals and observed to ensure lack of adverse clinical signs.

5.2 Animal Care and Maintenance

Housing: individually housed

Cages: suspended stainless steel

Bedding: hardwood chips

Animal room temperature: 68±5°F

Animal room relative humidity: 30-70%

Air exchanges per hour: 10 to 15

Lights: 12-hour light/dark cycle, full spectrum fluorescent lights

The laboratory and animal rooms were maintained as limited-access facilities.

Animal rations: commercial rabbit ration, *ad libitum*
Water: tap water, *ad libitum*

There was no known contaminants present in the feed, water, or bedding expected to interfere with the test data.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

6.1 Albino rabbits were used in this study because they have historically been used in safety evaluation studies. The guidelines offer no alternative (non-animal) methods. Dermal exposure corresponded to a likely route of human exposure. The species and number of animals used in this study are recommended by the ISO guidelines.

6.2 The test system was directly exposed to the test article. This is one route of administration available in this test system.

7.0 EXPERIMENTAL DESIGN

7.1 Pretreatment Screening Procedure
Animals selected for the test were examined to insure that their skin was free from irritation, trauma and disease.

7.2 Preparation of Test Animals
Two application sites for each test or control article were prepared by clipping the skin of the trunk free of hair within 24 hours before application of the test article. The sites of application were not abraded deliberately nor accidentally during preparation. The sites were covered with a 25 mm x 25 mm non-occlusive dressing and then wrapped with a semi-occlusive bandage for a minimum of 4 hours.

7.3 Post Treatment Procedures
At the end of the contact period, the dressings were removed and the skin wiped to remove any test article still remaining. Animals were observed for signs of erythema and edema at 30-60 minutes, and then at 24, 48, and 72 hours after bandage removal. Observations were scored according to the "Draize Scale for Scoring Skin Reactions" (see Appendix I).

7.4 Animals were observed daily for clinical manifestations.

7.5 Moribund and Dead Animals
All animals survived for the duration of the study.

7.6 Animals were weighed at the end of the observation period and returned to the general colony.

7.7 Preparation of Test and Control Article Extracts:
The test article was extracted in USP 0.9% Sodium Chloride for Injection (NaCl) and Cottonseed Oil (CSO). The test article was pre-soaked in the appropriate extraction media prior to the extraction procedure. The prepared test article was extracted in NaCl and CSO at a ratio of 120 cm^2 per 20 mL according to ISO 10993-12. Extractions were performed by heating the prepared test and control articles at 37°C for 24 hours. A control (20 mL) of each extracting medium was prepared for parallel treatments and comparisons. Each control was prepared in the same manner as the test article.

8.0 DOSAGE

A dose of 0.5 mL test or control article extract was applied to each application site. Separate animals were not required for an untreated control group. Each animal served as its own control.

8.2 Test and control article applications were as follows:

Cranial	
Clipped Dorsal Region	
Test Intact Site	Test Intact Site
Control Intact Site	Control Intact Site
Caudal	

9.0 EVALUATION CRITERIA

9.1 Evaluation of Animal Data
Observation values were calculated by averaging the scores for each of 3 individual animals. This was performed by adding the scores for each animal for erythema and edema at 24 hours, 48 hours, and 72 hours (at a minimum). This total was divided by 6 (2 test sites times 3 observation periods). A similar assessment was made of the control sites. The control score was subtracted from the test article score. Then, this calculated value for each animal was added together for a total of three animals. The total was divided by 3 to obtain the Primary Irritation Index.

9.2 Evaluation of Test Results
A test article with a Primary Irritation Index of less than 0.5 is considered a negligible irritant. Test article with indices of 0.5 to less than 2.0 is slight irritants. Test articles with indices of 2.0 to less than 5.0 are moderate irritants. Any test articles with an index of 5.0 or more are considered severe irritants. Dermal irritants are those test articles that produce reversible changes in the derma. Those test articles that destroy the structure of the intact skin or change it irreversibly are considered corrosive.

10.0 RESULTS

10.1 Body Weights (Table I)
All of the test animals exhibited a gain in body weight during the study.

10.2 Clinical Observations (Table I)
No overt signs of toxicity were evident in any of the animals during the course of the study.

10.3 Dermal Irritation (Table II)
No signs of erythema or edema were present at the 24, 48 or the 72 hour observation point. None of the control sites of any animal at any of the observation periods showed signs of erythema or edema.

10.4 Primary Dermal Irritation Index (PDII) (Table II)
PDII (Test article, NaCl) = 0
PDII (Test article, CSO) = 0

11.0 CONCLUSION

The test article, Hemostatic Agent (Sodium Chloride and Cottonseed Oil Extracts), was evaluated for its potential to produce Primary Dermal Irritation after a single topical 4 hour application to the skin of albino rabbits. The test article is considered a negligible irritant.

12.0 RECORDS

12.1 Original raw data will be archived at Toxikon Corporation.

12.2 A copy of the final report and any report amendments will be archived at Toxikon Corporation.

12.3 The original final report and a copy of any protocol amendments or deviations will be forwarded to the Sponsor.

12.4 All unused test article shall be discarded by Toxikon.

12.5 Final reports shall not be reproduced except in full, without the written authorization/approval from Toxikon

13.0 CONFIDENTIALITY AGREEMENT

Statements of confidentiality may be agreed upon prior to study initiation.

14.0 POLICY ON PAIN AND SUFFERING IN ANIMALS

No evidence of pain and suffering was observed in the animals.

15.0 ANIMAL USAGE

The Sponsor assures that to the best of their knowledge this study does not unnecessarily duplicate previous testing.

TABLE I

Animal Weights and Clinical Observations

Test Article: Hemostatic Agent **Date Administered:** 03/12/97

Lot #: 9702 **Animal Species:** Rabbit

Animal #	Sex	Body Weight (Kg) Day 0 03/12/97	Day 3 03/15/97	Weight Change	Signs of Toxicity*
70170	Female	2.66	2.69	0.03	None
70171	Male	2.70	2.74	0.04	None
70177	Male	2.78	2.81	0.03	None
70178	Female	2.79	2.83	0.04	None
70179	Male	2.74	2.76	0.02	None
70180	Female	2.78	2.84	0.06	None

* Excluding erythema and edema observations at 30-60 minutes after the 4 hour exposure, 24, 48 and 72 h.

TABLE II-A
DRAIZE SCORES
TEST SITES (NaCl Extract)

ANIMAL #	03/12/97 30-60 Minutes*		03/13/97 24 Hours		03/14/97 48 Hours		03/15/97 72 Hours		Total ** Divided by 6
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	
70170	0	0	0	0	0	0	0	0	0.0
70171	0	0	0	0	0	0	0	0	0.0
70177	0	0	0	0	0	0	0	0	0.0

** Primary Dermal Irritation Index (PDII) = 0.00

CONTROL SITES

ANIMAL #	03/12/97 30-60 Minutes*		03/13/97 24 Hours		03/14/97 48 Hours		03/15/97 72 Hours		Total Divided by 6
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	
70178	0	0	0	0	0	0	0	0	0.0
70179	0	0	0	0	0	0	0	0	0.0
70180	0	0	0	0	0	0	0	0	0.0

* 30-60 minute scores are not included in determining the PDII.
** Site 1/Site 2

TABLE II-B
DRAIZE SCORES
TEST SITES (CSO Extract)

ANIMAL #	03/12/97 30-60 Minutes*		03/13/97 24 Hours		03/14/97 48 Hours		03/15/97 72 Hours		Total ** Divided by 6
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	
70170	0	0	0	0	0	0	0	0	0.0
70171	0	0	0	0	0	0	0	0	0.0
70177	0	0	0	0	0	0	0	0	0.0

** Primary Dermal Irritation Index (PDII) = 0.00

CONTROL SITES

ANIMAL #	03/12/97 30-60 Minutes*		03/13/97 24 Hours		03/14/97 48 Hours		03/15/97 72 Hours		Total Divided by 6
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	
70178	0	0	0	0	0	0	0	0	0.0
70179	0	0	0	0	0	0	0	0	0.0
70180	0	0	0	0	0	0	0	0	0.0

* 30-60 minute scores are not included in determining the PDII.
** Site 1/Site 2

APPENDIX I: Draize Scale for Scoring Skin Reactions

Reaction	Value
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Total possible erythema score	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately lmm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Total possible edema score	4

TOXIKON FINAL REPORT: 97G-0348

SYSTEMIC INJECTION TEST - ISO

Author
Vasudev P. Anand, Ph.D.

Final Report Date
March 31, 1997

MANAGEMENT OF THE STUDY

Performing Laboratory
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor
Whalen Biomedical
11 Miller Street
Somerville, MA 02113

TABLE OF CONTENTS

STUDY SUMMARY

This test is considered negative based on standards set by the study protocol. Extracts of the test article, Hemostatic Agent, did not show a significantly greater biological reaction than the control extracts, when tested in albino Swiss mice.

QUALITY ASSURANCE STATEMENT

Based on a review of this study report, protocol and Standard Operating Procedures utilized, and inspection of study events listed below, this test conformed to the FDA Good Laboratory Practice (GLP) regulations, 21 CFR, Part 58.

The Sponsor was responsible for all test article purity, stability, and characterization data as specified in 21 CFR, Parts 58.105 and 58.113. The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED MANAGEMENT	DATE REPORTED STUDY DIRECTOR
CLINICAL OBSERVATIONS	03/17/97	03/17/97	03/17/97
RAW DATA	03/31/97	03/31/97	03/31/97
FINAL REPORT	03/31/97	03/31/97	03/31/97

Bernard A. Lech

Bernard A. Lech, M.S.
Quality Assurance Officer

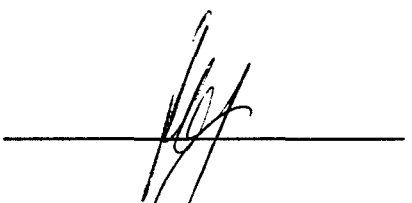
3/31/97
Date

STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

This study meets the technical requirements of the protocol. The study also meets with the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58.

Study Director: Vasudev P. Anand, Ph.D.

Company: Toxikon Corporation

Signature: 

Date: 3/31/97

Study Supervisor: Laurence Lister, B.S.

VERIFICATION DATES:

The study dates were as follows:

Protocol Effective Date:	02/05/97
Test Article Receipt:	02/25/97
Project Log Date:	02/25/97
Extraction Dates:	03/13/97 - 03/14/97
Technical Initiation:	03/14/97
Technical Completion:	03/17/97
Final Report Date:	03/31/97

1.0 PURPOSE

The purpose of the test is to screen solutions and test article extracts for potential toxic effects as a result of a single-dose systemic injection in mice.

2.0 REFERENCES

The test was conducted based upon the following references:

2.1 Biological Evaluation of Medical Devices - Part 11: Tests for Systemic Toxicity, ANSI/AAMI/ISO 10993-11:1993.

2.2 Biological Evaluation of Medical devices - Part 12: Sample Preparation and Reference Materials, ISO/CD (1993) 10993-12.

3.0 COMPLIANCE

The study conformed to all applicable laws and regulations. Specific regulatory requirements included the current FDA, 21 CFR, Part 58 - Good Laboratory Practice for Nonclinical Laboratory Studies; AAALAC, "Guide for the Care and Use of Laboratory Animals," National Research Council, 1996. (NIH) (OPRR), "Public Health Service Policy on Humane Care and Use of Laboratory Animals," Health Research Extension Act of 1985 (Public Law 99-158 November 20, 1985), Reprinted 1996; USDA, Department of Agriculture, Animal and Plant Health Inspection Service, 9 CFR Ch.1 (1/1/95) edition, Subchapter A-Animal Welfare. ISO 10993-2 (1993).

4.0 IDENTIFICATION OF TEST AND CONTROL ARTICLES

The following information was supplied by the Sponsor wherever applicable; it did not apply to confidential information. The Sponsor was responsible for all test substance characterization data as specified in the GLP regulations. Test and control articles (exclusive of extracts) that are mixed with carriers require verification of concentration, homogeneity and stability. Samples of test and control article mixtures will be returned to the Sponsor for characterization, wherever applicable.

4.1 Test Article
Test Article Name: Hemostatic Agent
CAS/Code: Not Supplied by Sponsor (N/S)
Lot/Batch: 9702
Physical State: Powder
Color: White
Density: N/S
Stability: Refrigerate

Solubility: N/S
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Procautions

4.2 Control Articles - (Toxikon Supplied)

4.2.1 Control Article Name: USP 0.9% Sodium Chloride Injection (NaCl)
QC Inventory #: CSC-96-10-005-VIV
Physical State: liquid
Color: Clear
Storage Conditions: Room temperature
Safety Precautions: Standard laboratory safety precautions apply

4.2.2 Control Article Name: Cottonseed Oil (CSO)
QC Inventory #: CSC-96-11-005-VIV
Physical State: liquid
Color: Yellow
Storage Conditions: Room temperature
Safety Precautions: Standard laboratory safety precautions apply

5.0 IDENTIFICATION OF TEST SYSTEM

5.1 Animals Used in the Test:

Number and species: 20 Albino Swiss Mice (Mus musculus)

Sex: female

Weight/Age range: 17-23 grams / 34-41 days old (adult)
Weighed to nearest 0.1 gm

Health Status: healthy, not previously used in other experimental procedures

Animal Identification: ear punch

Animal Purchase: Taconic

5.2 Animal Care and Maintenance:

Housing: group housed (5 mice of same sex)

Bedding: hardwood chips

Acclimatization: minimum 5 days under the same conditions as for the actual test.

Animal room temperature: 68±5°F

Animal room relative humidity: 30-70%

Air exchanges per hour: 10 to 15

Lights: 12-hour light/dark cycle, full spectrum fluorescent lights

Animal rations: Agway Prolab RMH 3000, *ad libitum*

Water: tap water, *ad libitum*

The laboratory and animal rooms were maintained as limited-access facilities.

Animal Selection: chosen from larger pool & examined for lack of adverse clinical signs

There were no known contaminants present in the feed, water, or bedding expected to interfere with the test data.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

6.1 Mice were used in this study because they have historically been used in systemic safety evaluation studies and the guidelines have no alternative (non-animal) methods. Animals were treated by intravenous and intraperitoneal routes. The animal species, number, and route of test article administration are recommended by the ISO 10993 guidelines.

6.2 The test article was administered through solutions compatible with the test system. This was the optimal route of administration available in this test system.

7.0 EXPERIMENTAL DESIGN

7.1 The animals were dosed as described in section 8.0 and observed for clinical signs immediately after injection, again 4 hours after injection, and then at 24, 48, and 72 hours after injection. Observations conducted included all clinical and toxicologic signs.

7.2 Animals were weighed at the end of the observation period and sacrificed by carbon dioxide (CO_2) inhalation.

8.0 DOSAGE

8.1 Preparation of Test Article Extracts
The test and control articles were prepared in ratios specified by ISO 10933.

8.1.1 The test article extracts were prepared with the following media:

A: USP 0.9% Sodium Chloride Solution for Injection (NaCl)
B: Cottonseed Oil (CSO)

Prior to extraction, the test material was pressed into a sheet of <0.5 mm thickness. Properly prepared test articles were extracted at a ratio of 120 cm^2 in 20 mL of the appropriate medium. The extraction medium completely covered the test article.

Each extracting medium (control article) was prepared for parallel treatments and comparisons. Each control article was prepared in the same manner as the test article.

8.1.2 The extracts were prepared at 37°C for 24 hours, per Sponsor specifications. Each extract was agitated vigorously prior to administration.

8.1.3 Groups of 5 animals were injected with either the test article extract or the corresponding control article extract in the same amounts and by the same routes set forth below:

Extract	Route	Dose/Kg	Injection Rate
NaCl	Intravenous	50 ml	0.1 ml/sec
CSO	Intraperitoneal	50 ml	-

9.0 EVALUATION CRITERIA

This test is considered negative if none of the animals injected with the test article shows a significantly greater biological reaction than the animals treated with the control article.

If two or more mice die, or show signs of toxicity such as convulsions or prostration, or if three or more mice lose more than 2 g of body weight, the test article does not meet the requirements of the test. If any animal treated with a test article shows only slight signs of biological reaction, and not more than one animal shows gross signs of biological reaction or dies, a repeat test should be conducted using groups of 10 mice. On the repeat test, all 10 animals must not show a significantly greater biological reaction than the animals treated

with the control article.

10.0 RESULTS

10.1 Body Weights: All of the test and control animals increased in weight.

10.2 Clinical Observations: None of the test or control animals exhibited overt signs of toxicity at any of the observation points.

10.3 The test is considered negative because none of the animals injected with the extract of the test article showed a significantly greater biological reaction than the animals treated with the control articles.

11.0 CONCLUSION

This test is considered negative based on standards set by the study protocol. Extracts of the test article, Hemostatic Agent, did not show a significantly greater biological reaction than the control articles, when tested in albino Swiss mice.

12.0 RECORDS

12.1 Original raw data is archived at Toxikon Corporation.

12.2 A copy of the final report and any report amendments is archived at Toxikon Corporation.

12.3 The original final report and a copy of any protocol amendments or deviations is forwarded to the Sponsor.

12.4 All unused test article shall be disposed of by Toxikon.

12.5 Final reports shall not be reproduced except in full, without the written authorization/approval from Toxikon.

13.0 CONFIDENTIALITY AGREEMENT

Statements of confidentiality were as agreed upon prior to study initiation.

14.0 POLICY ON PAIN AND SUFFERING IN ANIMALS

No evidence of pain and suffering were observed in the animals.

15.0 ANIMAL USAGE

The Sponsor assures that, to the best of their knowledge, this study did not unnecessarily duplicate previous testing.

TABLE I
Systemic Test
Animal Weights and Clinical Observations

Technical Initiation: 03/14/97 **Technical Completion:** 03/17/97

Test Article: Hemostatic Agent **Lot #:** 9702

Group	Sex	Dose (ml)	Animal #	Body Weight (g) Day 0 03/14/97	Body Weight (g) Day 3 03/17/97	Weight Change	Signs of Toxicity*
NaCl	Female	0.9	1	18.9	20.1	1.2	None
Test	Female	1.0	2	20.2	22.6	2.4	None
50ml/kg	Female	1.1	3	21.3	23.4	2.1	None
	Female	0.9	4	18.9	21.0	2.1	None
	Female	1.0	5	19.2	21.8	2.6	None
			Mean	19.7	21.8		
			SD +/-	1.0	1.3		
NaCl	Female	1.0	6	20.1	22.6	2.5	None
Control	Female	1.0	7	20.2	22.2	2.0	None
50ml/kg	Female	0.9	8	18.6	20.7	2.1	None
	Female	1.0	9	19.2	21.6	2.4	None
	Female	1.0	10	20.3	22.0	1.7	None
			Mean	19.7	21.8		
			SD +/-	0.7	0.7		
CSO	Female	1.0	11	20.6	22.4	1.8	None
Test	Female	1.1	12	21.3	23.0	1.7	None
50ml/kg	Female	1.0	13	20.2	22.6	2.4	None
	Female	1.1	14	21.1	23.4	2.3	None
	Female	1.0	15	20.8	22.0	1.2	None
			Mean	20.8	22.7		
			SD +/-	0.4	0.5		
CSO	Female	0.9	16	18.7	20.4	1.7	None
Control	Female	1.0	17	19.5	21.7	2.2	None
50ml/kg	Female	1.0	18	20.2	22.8	2.6	None
	Female	1.1	19	21.7	23.6	1.9	None
	Female	1.0	20	20.3	22.5	2.2	None
			Mean	20.1	22.2		
			SD +/-	1.1	1.2		

* Summary of observations - Immediate, 4, 24, 48, and 72 h after injection

TOXIKON FINAL REPORT: 97G-0345

COMPLEMENT ACTIVATION ASSAY-ISO

Author
Vasudev P. Anand, Ph.D.

Final Report Date
March 31, 1997

MANAGEMENT OF THE STUDY

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Graph II: SC5B
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Appendix II: SC5b-9 Enzyme Immunoassay

STUDY SUMMARY

The extract of the test article, Hemostatic Agent, was tested for its capacity to induce complement activation of C3 and C5 proteins in human plasma. Based upon the criteria of the study protocol, the test article induced complement activation of C3 and C5 proteins in human plasma as compared to both untreated plasma (plasma exposed to extraction vehicle - USP Sodium Chloride for Injection - NaCl) and negative control plasma (plasma exposed to the extract of negative control article - USP Plastic RS). Based on the criteria of the protocol, the test article does not meet the requirements of the complement activation assay - ISO, under the experimental condition employed.

QUALITY ASSURANCE STATEMENT

Based on a review of this study report, protocol and Standard Operating Procedures utilized, and inspection of study events listed below, this test conformed to the FDA Good Laboratory Practice (GLP) regulations, 21 CFR, Part 58.

The Sponsor was responsible for all test article purity, stability, and characterization data as specified in 21 CFR, Parts 58.105 and 58.113. The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED MANAGEMENT	DATE REPORTED STUDY DIRECTOR
SCORING	03/21/97	03/21/97	03/21/97
RAW DATA	03/31/97	03/31/97	03/31/97
FINAL REPORT	03/31/97	03/31/97	03/31/97

Bernard A. Lech, M.S.
Quality Assurance Officer

3/31/97
Date

STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

This study meets the technical requirements of the protocol. The study also meets with the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58.

Study Director: Vasudev P. Anand, Ph.D.

Company: Toxikon Corporation

Signature: _____

Date: 3/31/97

Study Supervisor: Mark Turner, B.S.

VERIFICATION DATES:

The study dates were as follows:

Protocol Effective Date:	02/05/97
Test Article Receipt:	02/25/97
Project Log Date:	02/25/97
Extraction Dates:	03/20/97-03/21/97
Technical Initiation:	03/21/97
Technical Completion:	03/21/97
Final Report Date:	03/31/97

1.0 PURPOSE

The activation of cascades of proteins down either the classical or alternative biological pathway is an important component in the assessment of immune responses to bacterial or viral infections. Activation of these cascades of proteins, especially C3 and C5, results in the generation of activation fragments (C3a and C5a), which in turn are responsible for the release of histamine from mast cells and basophils, contraction of smooth muscle, and increase in capillary permeability. Release of these peptides in sufficient quantity also can result in anaphylaxis in humans.

This *in vitro* assay is designed to measure complement activation in human plasma as a result of exposure of the plasma to the test article. The measure of complement activation indicates whether a test article is capable of inducing a complement - induced inflammatory immune response in humans.

2.0 REFERENCES

The assay was conducted based on the following:

2.1 Biological Evaluation of Medical Devices-Part 4: Selection of Tests for Interactions with Blood, ANSI/AAMI/ISO 10993-4: 1993; and EN 30993-4 (1994).

2.2 Burd, J.F., Noetzel, V. and Tamerius, J.D., Rapid Testing of Biomaterials for Complement Activation Using *In Vitro* Complement Immunoassays: 19th Annual Meeting of the Society for Biomaterials, April 1993.

2.3 C3a and SC5b-9 EIA Package Inserts, QUIDEL Corp., San Diego, CA

2.4 Extraction procedures, if applicable, was based upon the standard titled Biological Evaluation of Medical Devices-Part 12: Sample Preparation and Reference Materials, ISO/CD (1993) 10993-12.

2.5 Chenoweth D.E., Complement Activation Produced by Biomaterials Trans Am. Soc. Artif. Intern. Organs, Vol. 22, 226-232, 1986.

3.0 COMPLIANCE

The study conformed to all applicable laws and regulations. Specific regulatory requirements include the current FDA, 21 CFR, Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies.

4.0 IDENTIFICATION OF TEST AND CONTROL ARTICLES

4.1 The following information was supplied by the Sponsor wherever applicable; it does not apply to confidential information. The Sponsor was responsible for all test substance characterization data as specified in the GLP regulations. Test and control articles (exclusive of extracts) that are mixed with carriers require verification of concentration, homogeneity and stability. Samples of test and control article mixtures will be returned to the Sponsor for characterization, if required.

Test Article Name: Hemostatic Agent
CAS/Code #: Not Supplied by Sponsor (N/S)
Lot/Batch #: 9702
Physical State: Powder
Color: White
Density: N/S
pH: N/S
Stability: Refrigerate
Solubility: N/S
Expiration Date: 08/31/97
Safety Precautions: Standard Laboratory Safety Precautions

Negative Control Article: USP Plastic RS
QC #: CSC9603002VIV
Physical State: Solid
Color: Opaque
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

Negative Control Article: USP 0.9% Sodium Chloride for Injection (NaCl)
QC #: CSC9606003VIV
Physical State: Liquid
Color: Clear
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

Positive Control Article #1:
Control Article Name: Latex
QC #: CSC9504004CC
Physical State: Solid
Color: Yellow
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

Positive Control Article #2:
Control Article Name: Cellulose Acetate
QC #: CSC9405002CC
Physical State: Solid
Color: White
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

5.0 IDENTIFICATION OF TEST SYSTEM

5.1 The test system was human plasma. The plasma was from anonymous pre-screened donors from Children's Hospital Medical Center, Boston, MA.

5.2 C3a is a protein in human plasma that is generated during activation of the complement system. The anaphylatoxin C3a itself is very short-lived and in the serum is cleaved immediately into the more stable C3a-desArg. Therefore, quantitation of C3a-desArg allows reliable conclusions about the level of complement activation in the test plasma.

5.3 The C5 complement protein is cleaved into C5a and C5b. C5a is even more short-lived than C3a and is cleaved quickly. The C5b fragment in turn forms the SC5b-9 complex. Therefore, quantitation of C5a is possible by determining the concentration of SC5b-9 present in plasma exposed to the test article. The QUIDEL SC5b-9 enzyme immunoassay measures the concentration of SC5b-9 in human plasma. It uses a monoclonal antibody to a neoantigen in the complex to capture the SC5b-9 macromolecule.

5.4 The enzyme immunoassay kits utilized in this assay were obtained from Quidel Corporation, San Diego, CA.

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

The test article was administered *in vitro*, via a vehicle compatible with the test system, per Sponsor specification. This was the optimal route of administration available for this test system.

7.0 EXPERIMENTAL DESIGN

7.1 General Procedure for C3a (refer Appendix I):
The assay was performed using a kit obtained from QUIDEL Corp., San Diego, CA. Included in the kit is a microassay plate coated with a monoclonal antibody specific to a neoantigen for human C3a-desArg. For convenience, the neoantigen will be referred to in this protocol as C3a.

Plasma exposed to the test article extract at 37±2°C for 90 minutes was diluted 1:100 and delivered to the plate (100 uL/well), in triplicate. The plate was incubated to allow the C3a in the test article to bind to the monoclonal antibody.

Unbound native C3 was rinsed off and horseradish peroxidase (HRP)-conjugated rabbit anti-C3a was used for the detection of bound C3a. Excess conjugate was removed and the amount of C3a in the plasma was quantified using the peroxidase reaction and a standard curve, using a spectrophotometric method, at 450 nm.

7.2 General Procedure for SC5b-9 (refer Appendix II):
The SC5b-9 enzyme immunoassay is very similar to the C3a assay. It involves a three-step procedure utilizing (1) a microassay plate coated with a mouse monoclonal antibody which binds specifically to SC5b-9, (2) HRP-conjugated antibodies to antigens of SC5b-9, and (3) a chromogenic substrate.

In step (1), standards, controls and plasma exposed to the test article extract was added to microassay wells pre-coated with an anti-SC5b-9 specific monoclonal antibody. Any SC5b-9 present will bind to the immobilized anti-SC5b-9. Post-incubation, a wash cycle removed the unbound material.

In step (2), horseradish peroxidase (HRP)-conjugated antibodies to antigens on SC5b-9 was added to each test well. The enzyme-conjugated antibodies will bind to SC5b-9, which was captured by the monoclonal anti-SC5b-9 bound on the surface of the microassay wells. After incubation, a wash cycle removed unbound conjugate.

In step (3), a chromogenic enzyme substrate was added to each microassay well. The bound HRP-conjugate will react with the substrate turning a green color. After incubation, a reagent was added to stop color development, and the absorbance of the standard, controls and test article was measured spectrophotometrically at 405 nm. The color intensity of the reaction mixture is proportional to the concentration of SC5b-9 present.

8.0 DOSAGE

8.1 Per sponsor specification, the test article was extracted in USP 0.9% Sodium Chloride for Injection (NaCl) at a ratio of 120 cm^2/20 mL at 37°C for 24 hours. The positive control for the C3a assay (latex rubber) and the negative control article (USP Plastic RS) were extracted in NaCl at a ratio of 60 cm^2/20 mL, at 37°C for 24 hours. The positive control for the SC5b-9 assay, Cellulose Acetate, was extracted at a ratio of 3g/20 ml in NaCl at 37°C for 24 hours. The extracts were exposed to plasma (collected with citrate as anticoagulant) at a ratio of 1:10 (10% v/v). Plasma exposed to the extraction vehicle (NaCl) at 10% (1:10 volume:volume) was designated as the untreated plasma.

8.2 All other test article preparation was as specified by the Sponsor.

9.0 EVALUATION CRITERIA

9.1 A standard curve was obtained by plotting the absorbance values on the y-axis for each C3a and SC5b-9 concentration indicated on the x-axis. The concentration of C3a and SC5b-9 activated by the extracts of test article and controls was determined by reading the concentration from the x-axis corresponding to its absorbance (y- axis) value.

9.2 The assay is considered valid if a standard dose response curve is achieved with a correlation coefficient, $r \geq 0.95$.

9.3 The concentration of C3a and SC5b-9 in plasma, at each time point, and the significance ($p \leq 0.05$) as compared to that of the corresponding untreated plasma and plasma exposed to the extract of negative control article was determined.

9.4 The concentration of C3a and SC5b-9 in plasma exposed to the appropriate positive control article(s) extract should be significantly greater than the concentration of C3a and SC5b-9 in both the corresponding untreated and negative control plasma (extract of USP Plastic RS) for at least one of the time points (significant at $p \leq 0.05$).

9.5 The test article does not meet the requirements of the C3 or C5 protein activation test if the concentration of C3a or SC5b-9 protein is significantly higher than that in both negative controls, i.e., untreated plasma (plasma exposed to extraction vehicle, NaCl) and negative control plasma (plasma exposed to the extract of negative control article-USP Plastic RS) (significant at $p \leq 0.05$). Biological and Statistical Significance will be considered in the evaluation of the results.

10.0 CALCULATION

A standard curve was obtained by plotting the absorbance values on the y-axis for each C3a and SC5b-9 concentration indicated on the x-axis. The concentration of C3a and SC5b-9 activated by the test article and the control article was determined by reading the concentration from the x-axis corresponding to its absorbance (y-axis) value. Data analysis was performed by using Microsoft Excel and the student t-test. Differences between the groups were considered statistically significant only if the probability of the differences between the means being due to chance were equal to or less than 5% ($p \leq 0.05$).

11.0 RESULTS

11.1 C3a Assay

The standard curve for the C3a protein concentration (ng/ml) versus absorbance (O.D. at 450 nm) had a slope of 0.0009, and correlation coefficient of r=0.976.
The values for the C3a concentration in the treated and untreated plasma were determined based upon the standard curve. The results are presented in Table I and Graph I. The plasma exposed to the test article extract had a statistically significant increase in C3a as compared to the untreated plasma and negative control plasma at the 90 minute time point. The results indicated that the positive control had a statistically significant increase in C3a as compared to both negative control plasma at the 90 minute time point.

11.2 SC5b-9 Assay

The standard curve for the SC5b-9 protein concentration (ng/ml) versus absorbance (O.D. at 405 nm) had a slope of 0.006, and correlation coefficient of r=0.993.
The values for the SC5b-9 concentration in the treated and untreated plasma were determined based upon the standard curve. The results are presented in Table II and Graph II. The SC5b-9 concentration in plasma exposed to the test article was statistically significantly higher as compared to the untreated plasma and negative control plasma at the 90 minute time point. The results indicated that the positive control had a statistically significant increase in SC5b-9 as compared to both negative control plasma in at the 90 minute time point.

12.0 CONCLUSION

The extract of the test article, Hemostatic Agent, was tested for its capacity to induce complement activation of C3 and C5 proteins in human plasma. Based upon the criteria of the study protocol, the test article induced complement activation of C3 and C5 proteins in human plasma as compared to both untreated plasma (plasma exposed to extraction vehicle - USP Sodium Chloride for Injection - NaCl) and negative control plasma (plasma exposed to the extract of negative control article - USP Plastic RS). Based on the criteria of the protocol, the test article does not meet the requirements of the complement activation assay - ISO, under the experimental condition employed.

13.0 RECORDS

13.1 Original raw data is archived at Toxikon Corporation.

13.2 A copy of the final report and any amendments is archived at Toxikon Corporation.

13.3 The original final report and a copy of any protocol amendments or deviations is forwarded to the Sponsor.

13.4 All unused test article shall be discarded, per sponsor specification.

13.5 Final reports shall not be reproduced except in full, without the written authorization/approval from Toxikon.

14.0 CONFIDENTIALITY AGREEMENT

Statements of confidentiality were as agreed upon prior to study initiation.

TABLE I
C3a

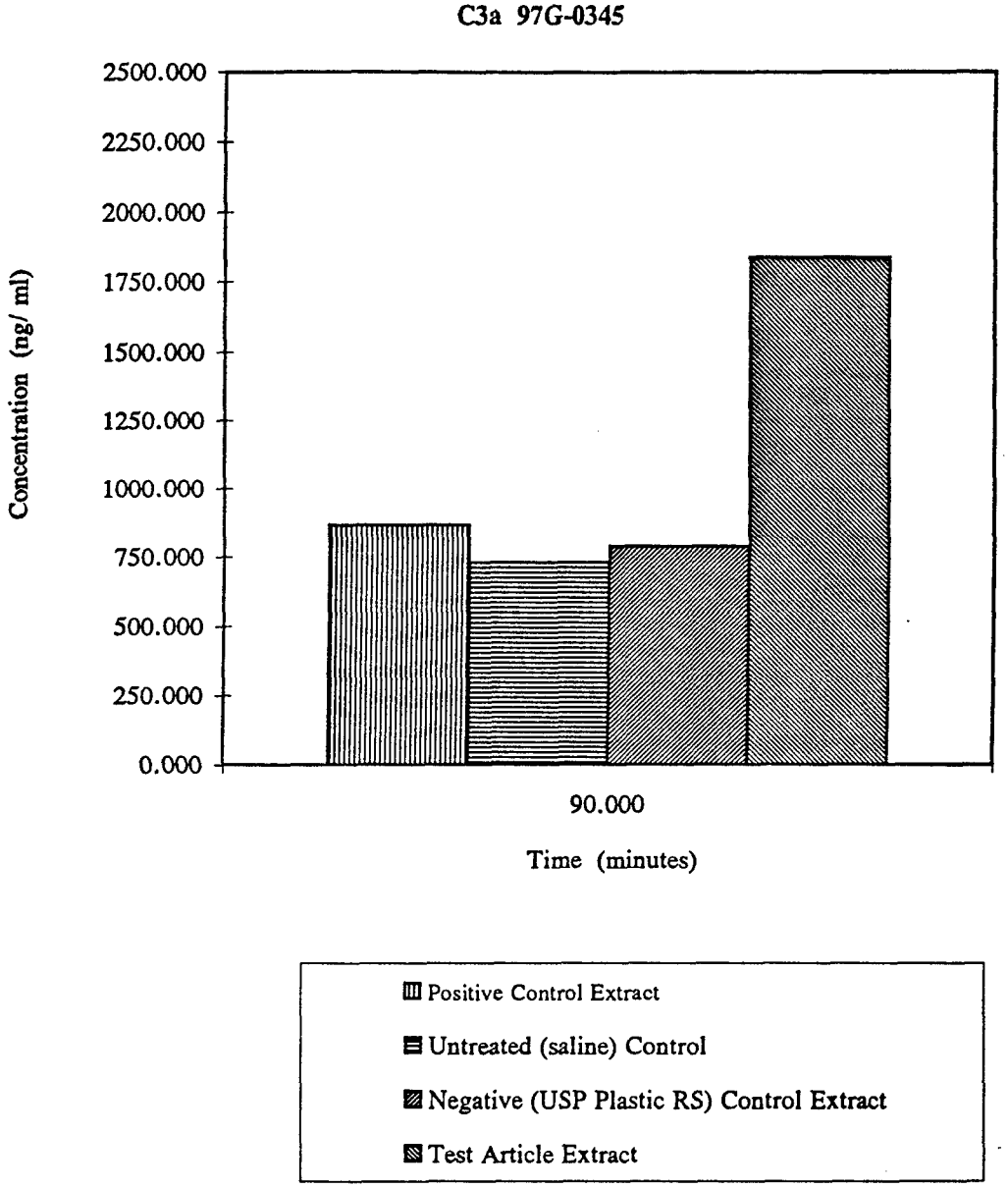
Sample	90 min
Untreated Plasma	729.8
Negative Control[a]	789.7
Positive Control[b]	868.5+@
Test Article	1837.5+@

TABLE II
SC5b-9

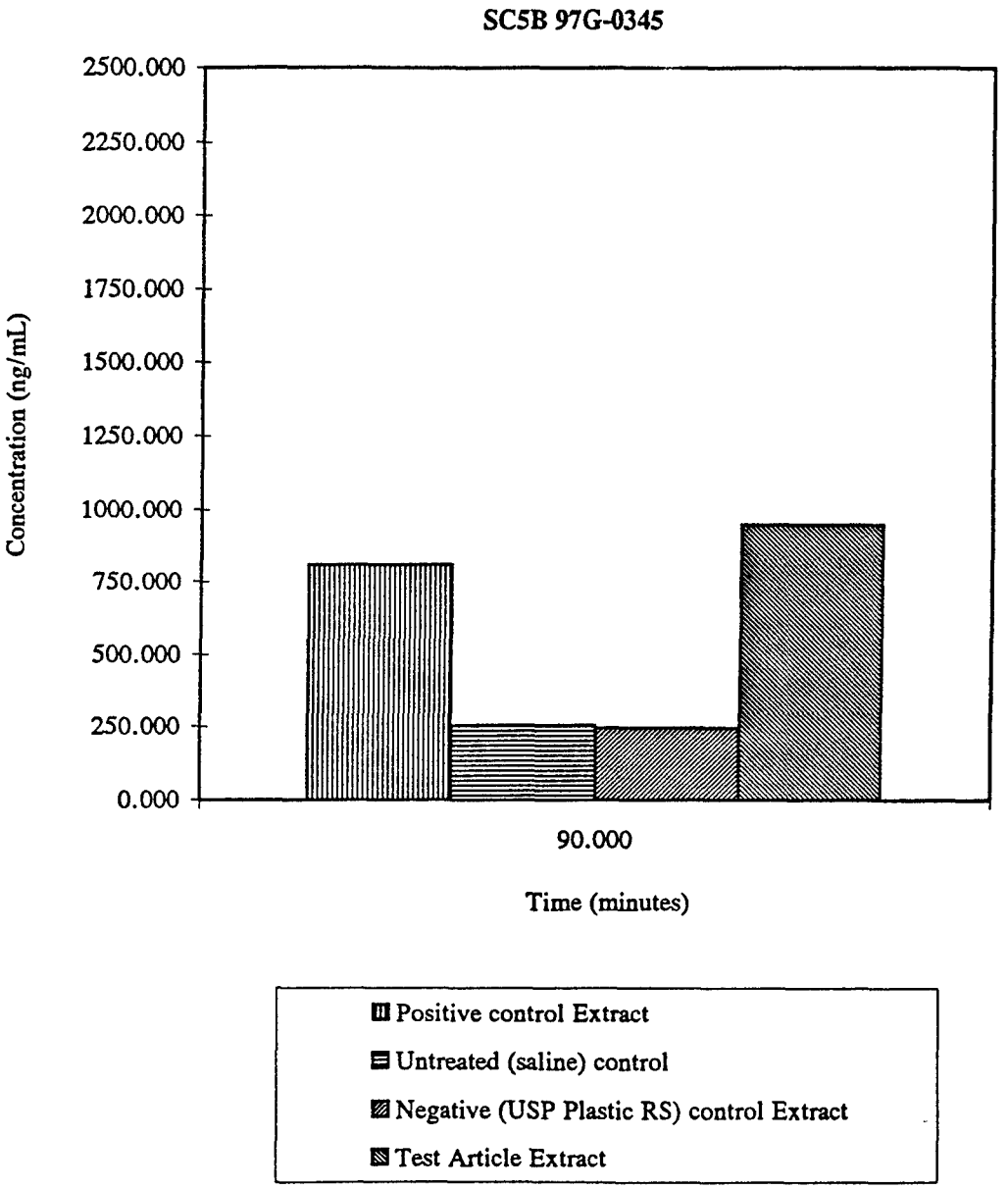
Sample	90 min
Untreated Plasma	254.2
Negative Control[a]	246.5
Positive Control[c]	809.3+@
Test Article	947.2+@

* Length of Exposure (minutes)
[a]Negative Control Article = USP Plastic RS (60 cm^2/20 mL)
[b]Positive Control = Latex Rubber (60 cm^2 /20 ml)
[c]Positive Control = Cellulose Acetate (3g/20 mL)
+ Significantly higher than untreated control plasma
@ Significantly higher than negative control plasma
# Statistically but not Biologically significant

GRAPH I


C3a 97G-0345
2500.000
2250.000
2000.000
1750.000
1500.000
1250.000
1000.000
750.000
500.000
250.000
0.000
Concentration (ng/ ml)
90.000
Time (minutes)
Positive Control Extract
Untreated (saline) Control
Negative (USP Plastic RS) Control Extract
Test Article Extract

GRAPH II


SC5B 97G-0345
2500.000
2250.000
2000.000
1750.000
1500.000
1250.000
1000.000
750.000
500.000
250.000
0.000
Concentration (ng/mL)
90.000
Time (minutes)
Positive control Extract
Untreated (saline) control
Negative (USP Plastic RS) control Extract
Test Article Extract

APPENDIX I
C3a ENZYME IMMUNOASSAY

1.0 PURPOSE

The purpose of this assay was to quantify the amount of C3a complement activated in plasma exposed to the test article.

2.0 TECHNICAL PROCEDURE

2.1 Separation of Plasma:
Citrated human plasma was obtained from anonymous donors at Children's Hospital Medical Center, Boston, MA., and immediately frozen at -70°C or below.

2.2 Exposure To Test Article:
The test article extract was exposed to plasma at 37±2°C as specified in section 8.0. Aliquots of the plasma were analyzed for the generation of C3a at one fixed interval 90 minutes. Plasma control tubes (untreated, negative and positive control plasma) were similarly prepared and incubated.

2.3 C3a Standard:
C3a Standard was dissolved in 1.0 mL of Sample Buffer (previously prepared per manufacturer's recommendations), mixed well, and incubated for not less than five minutes at room temperature. Serial dilutions (1:2, 1:4 and 1:8) of the Standard were prepared utilizing Sample Buffer as diluent. Reconstituted Standard was prepared fresh on the day of the assay and was discarded after use.

2.4 Plate Incubation:
The following was pipetted into the microassay plate (100 uL/well), in triplicate:

* Untreated Sample Buffer (Blank)
* C3a Standard Neat
* C3a Standard 1:2
* C3a Standard 1:4
* C3a Standard 1:8
* Negative Control (Quidel; consists of lyophilized human plasma with C3a content <200 ng/mL)
* Untreated Plasma (plasma incubated at 1 time interval and exposed to the extraction vehicle)
* Negative Control Plasma (plasma exposed to the extract of negative control article [USP Plastic RS] at 1 time interval)
* Treated Plasma (plasma exposed to test article extract at 1 time interval)

* Positive Control Plasma (plasma exposed to the extract of latex tubing at 1 time interval)
All test and control plasma were diluted 1:100 by adding 10 uL of the plasma to 1.0 mL of prepared Sample Buffer, 1 time interval)

The plate was incubated at room temperature for 60±2 minutes.

2.5 Wash:
The microassay plate was emptied and filled with Wash Buffer, previously prepared according to manufacturer's recommendations, (200 uL/well). After 60±2 seconds, the plate was emptied and this wash procedure repeated twice more. Excess wash liquid was removed by tapping the strips onto absorbent paper.

2.6 Conjugate Incubation:
C3a Conjugate (100 uL) was pipetted into each well and incubated at room temperature for 60±2 minutes. The microassay plate was emptied and the wash procedure in section 2.5 repeated.

2.7 Substrate Incubation:
Substrate solution (prepared per manufacturer's recommendations) was pipetted into each well (100 uL/well) and incubated at room temperature for 15±2 minutes.

2.8 Stop Reaction:
Stop Solution was pipetted into each well (100 uL/well) using the same sequence utilized when adding the substrate.

2.9 Absorbance:
The absorbance was measured with a microassay plate reader at 450 nm.

Note: The absorbance for the buffer blank was <0.2 and the neat Standard was >0.5 at 450 nm. Therefore, the assay was working properly.

3.0 REFERENCE

C3a EIA Package Inserts, QUIDEL Corp., San Diego, CA

APPENDIX II
SC5b-9 ENZYME IMMUNOASSAY

1.0 PURPOSE

The purpose of this assay was to quantify the amount of SC5b-9 complement activated in plasma exposed to the test article.

2.0 TECHNICAL PROCEDURE

2.1 Separation Of Plasma:
Citrated human plasma was obtained from anonymous donors at Children's Hospital Medical Center, Boston, MA., and immediately frozen at -70°C or below.

2.2 Exposure To Test Article:
The test article extract was exposed to the plasma as specified in section 8.0 at 37±2°C. Aliquots of the plasma were analyzed for the generation of SC5b-9 at one fixed interval, 90 minutes. Plasma control tubes (untreated, negative control and positive control plasma) were similarly incubated.

Plasma samples were diluted at a ratio of 1:4, in Complement Specimen Diluent. The dilution is required so that the absorbance values (at 405 nm) observed for the treated plasma do not exceed the absorbance values of the SC5b-9 kit Standard C and are at least 0.150 units above the background blank reading.

2.3 SC5b-9 Standards:
The SC5b-9 Standards A, B and C are part of the QUIDEL kit. Each standard consists of human serum containing known amounts of SC5b-9.

2.4 Additional Controls:
SC5b-9 High Control (Human) contains human serum with a high level of SC5b-9 complexes, while SC5b-9 Low Control (Human) contains low levels of the same complexes. Each control will be diluted 1:25 in Complement Specimen Diluent.

2.5 Analysis:
2.5.1 The microassay plate was rehydrated by adding 300 uL of Wash Solution (previously diluted per manufacturer's directions) to each well. The plate was incubated at room temperature for two minutes. The liquid was removed from each well and the plate inverted and tapped firmly on absorbent paper twice to remove any remaining liquid.

2.5.2 The following were pipetted into the microassay plate (100 uL/well):
* Complement Specimen Diluent (Blank) in triplicate
* SC5b-9 Standard A in triplicate
* SC5b-9 Standard B in triplicate
* SC5b-9 Standard C in triplicate

* SC5b-9 High Control (1:25 dilution) in triplicate
* SC5b-9 Low Control (1:25 dilution) in triplicate
* Untreated Plasma (plasma incubated at 1 time interval exposed and exposed to extraction vehicle) in triplicate
* Treated Plasma (plasma exposed to test article extract at 1 time interval) in triplicate
* Positive Control Plasma (plasma exposed to the extract of Cellulose Acetate at 1 time interval) in triplicate
* Negative Control Plasma (plasma exposed to the extract of negative control article [USP Plastic RS] at 1 time point) in triplicate

The plate was incubated at room temperature for 60±2 minutes.

2.5.3 Wash:
The microassay plate was emptied and filled with Wash Solution, previously prepared according to manufacturer's recommendations, (300 uL/well). After 60±2 seconds, the plate was emptied and this wash procedure repeated four times more. Excess wash liquid was removed by tapping the strips onto absorbent paper.

2.5.4 Conjugate Incubation:
SC5b-9 Conjugate (50 uL) was pipetted into each well and incubated at room temperature for 60±2 minutes. The microassay plate was emptied and the wash procedure in section 2.5.3 repeated.

2.5.5 Substrate Incubation:
Substrate Solution (prepared fresh per manufacturer's directions) was pipetted into each well (100 uL/well) and incubated at room temperature for 30±1 minutes.

2.5.6 Stop Reaction:
Stop Solution was pipetted into each well (50 uL/well) using the same sequence used when adding the substrate. The plate was tapped gently to disperse the color development evenly.

2.5.7 Absorbance:
The absorbance of each well was measured with a microassay plate reader at 405 nm immediately after the addition of Stop solution.

3.0 REFERENCE

SC5b-9 EIA Package Inserts, QUIDEL Corp., San Diego, CA

Appendix C

U.S. Patent No. 5,464,471

Fibrin Monomer Based Tissue Adhesive

US005464471A

United States Patent [19]

Whalen et al.

[11] Patent Number: 5,464,471

[45] Date of Patent: Nov. 7, 1995

[54] **FIBRIN MONOMER BASED TISSUE ADHESIVE**

[75] Inventors: **Robert L. Whalen**, Somerville; **Donald Dempsey**, Newbury, both of Mass.

[73] Assignee: **Whalen Biomedical Inc.**, Somerville, Mass.

[21] Appl. No.: **339,176**

[22] Filed: **Nov. 10, 1994**

[51] **Int. Cl.**[6] **C09J 189/00**

[52] **U.S. Cl.** **106/124**; 604/290; 514/12

[58] **Field of Search** 106/124; 606/214; 604/290; 514/12

[56] **References Cited**

U.S. PATENT DOCUMENTS

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4,362,567	7/1982	Schwarz et al.	106/157
4,414,976	11/1983	Schwarz et al.	106/161
4,735,616	4/1988	Eibl et al.	606/191
4,909,251	3/1990	Seelich	606/213
5,219,895	6/1993	Kelman et al.	522/68
5,330,974	7/1994	Pines et al.	514/21

Primary Examiner—David Brunsman

[57] **ABSTRACT**

A single agent fibrin based hemostatic and tissue adhesive agent comprised of recombinant fibrin monomer, Bovine thrombin, and calcium chloride, which is lyophilized to powder form, and is activated upon blood contact, at the site of tissue injury, producing effective hemostasis and subsequent tissue adhesion.

7 Claims, No Drawings

FIBRIN MONOMER BASED TISSUE ADHESIVE

LICENSE RIGHTS

The U.S. government has a paid up license in this invention and the right, in limited circumstances to require the patent owner to license others on reasonable terms as provided by U.S. Army contract number DAMD 12-94-C-4039.

BACKGROUND

1. Field of the Invention

This invention relates to a fibrin based tissue adhesives for achieving hemostasis.

2. Description of Prior Art

The ability to establish expeditious and permanent bonding between biological tissues is a critical factor in the success of many medical procedures, from surgical operations to wound dressings. Attempts to provide such bonding through mechanical means have proven inadequate. Consequently, biomedical research has focused on the development of natural and synthetic materials to act as adhesives, sealants and hemostatic agents. In connection with the description of previous inventions and the invention herein, the terms adhesive, sealant and hemostatic agent are defined broadly and used as these terms are understood in the art.

The clinical applications of such tissue adhesive agents are extensive and diverse. Aside from simple hemorrhage control and wound closure, applications include the treatment and preservation of the ruptured spleen (Brands, W. et al., World J. Surg., 6. 366–368, (1982)), the sealing of vascular prostheses (Walterbusch. G. et al., Thorac. cardiovasc. Surgeon, 30, 234–235, (1982)), the sealing of vascular grafts prior to implantation (Kalmer, P. et al., Thorac. cardiovasc. Surgeon, 30, 230–23 1, (1982)), the sealing of microvascular anastomoses (Pearl, RM et al., Surgery, Gynecology & Obstetrics, 144, 227–230, (1977)), the repair of middle ear defects (Epstein, GH et al., Ann Otol. Rhinol. Laryngol., 95, 40–45, (1986), and Silberstein. LE et al., Transfusion, 28(4), 319–321, (1988)), and the bonding of a corneal inlay into a recess prepared to receive same in the cornea of a patient. In fact, marketing research has indicated that there are over 8 million surgical procedures that could utilize a safe, effective biological adhesive.

Major interest in the use of synthetic polymeric materials to act as adhesives, sealants and hemostatic agents began in the early sixties. Initial work was confined to water-soluble systems such as casein and polyvinyl alcohol, but was later expanded to include all available synthetic adhesives and plastics with no known local or general toxicity. Although many materials were investigated, the most widely used tissue adhesives were the cyanoacrylates. These are a homologous series of organic molecules which polymerize and adhere to moist living tissues. Methyl-alpha-cyanoacrylate (MCA) in particular, has been used since 1960 by many investigators as a non-suture adhesive for bones. MCA is a fluid, monomeric material which polymerizes in seconds under mild pressure to produce a thin, strong, adherent film. However, these materials have been shown to be histotoxic and induce detrimental inflammatory tissue reaction.

Such toxicity with synthetic adhesives has led investigations toward the development of biologically derived bonding materials. These materials often consist of elements from

the natural bonding mechanism, such as collagen or fibrin. Collagen is a major connective tissue protein which is evident in prior art of many biomedical products, such as an artificial cornea (U.S. Pat. No. 4,581,0303, a hemostatic agent (U.S. Pat. No. 4,2 15,2003 and a soft contact lens (U.S. Pat. Nos. 4,264,155: 4,264,493; 4,349,470; 4,388,428; 4,452,925 and 4,650,6163, due to its good biocompatibility. However, natural collagen must be modified to render it suitable for use as a biomedical adhesive.

In many instances, the prior modified collagen-based adhesives suffer from various deficiencies which include (1) crosslinking/polymerization reactions that generate exothermic heat, (2) long reaction times, and (3) reactions that are inoperative in the presence of oxygen and physiological pH ranges (Lee, ML et al., Adhesion in Biological Systems, RS Manly, ed., Academic Press, New York, 1970. Ch. 17) Moreover, many of these adhesives contain toxic materials rendering them unsuitable for biomedical use. As a result, recent processing developments have been revealed, as in U.S. Pat. No. 5,219,895 to Kelman, in which pure, soluble or partially fibrillar collagen monomers are chemically modified to be soluble at physiological conditions and polymerize to achieve sealant properties.

Improvements of this sort still have not produced an agent as effective in achieving hemostasis as fibrin based tissue adhesives. Compared with oxidized cellulose, microfibrillar collagen, or surface charge modified collagen, the use of fibrin adhesive results in significantly less blood loss at the site of injury (Raccuia, JS et al. Comparative efficacy of topical hemostatic agents in a rate kidney model. Am. J. Surg. 163(2):234–8, 1992). Consequently, tissue adhesives of this type have been developed as seen in U.S. Pat. Nos. 4,362,567 and 4,414,976 and Can. Pat. No. 1,168,982. The foundation of these agents are the proteins fibrinogen and thrombin.

Fibrinogen is a soluble protein found in the blood plasma of all vertebrates. When fibrinogen is contacted by thrombin, a protein enzyme, it is converted into fibrin monomer and Factor XIII is activated to Factor XIIIa. Factor XIIIa then polymerizes the fibrin monomer to form a stabilized fibrin network. Such a network is essential to the healing process of wound closure and tissue bonding. The network serves as a physical bond and as a scaffolding to support the migration of immunologically active cells, for defense against invading pathogens, and epithelial cells, for tissue regeneration and repair. The fibrin network may then be gradually dissolved by the body (fibrinolysis) after treatment leading to a more normal appearance of the healed site.

Tissue adhesive preparations of this type usually consist of a fibrinogen solution containing Factor XIII, some additional proteins, such as fibronectin and albumin, and active or nonactive additions. A thrombin solution may also be provided containing thrombin and calcium ions, or the thrombin may be provided from the tissue area to be bonded itself. These solutions are commercially available in the form of either deep-frozen solutions or lyophilisate due to their lack of stability as liquid aqueous solutions. Therefore, these products are typically packaged in the form of kits, which include the protein ingredients, means to prepare the solutions, and means to utilize the solutions.

In emergency situations, quick availability of the tissue adhesive may be of decisive importance. However, the use of these kits is often difficult, tedious and time consuming. These constraints pose a problem in the hospital setting and may be completely defeating in the field or combat settings. To overcome this, there have been attempts to shorten the

preparation time. This usually involves shortening the reconstitution time of lyophilized solutions. For instance, Can. Pat. No. 1,182,444 describes a method and an arrangement for accelerating the dissolution of lyophilized medicines. The combined heating and stirring device disclosed markedly shortened reconstitution times, yet physicians have voiced a desire for further improvements.

It has been known that the solubility of hard-soluble proteins can be improved by certain additions. Thus, EP-A-0 085 923 discloses a lyophilized fibrinogen composition which additionally contains a further substance having a urea or guanidine residue. However, it has been shown that such additions have a cytotoxic effect, inhibit the growth of fibroblasts and cause the formation of an irregular fibrin structure resulting in the loss of desired elasticity of the fibrin. These effects jeopardize the desired properties of fibrinogen-based tissue adhesives, such as the stimulation of wound healing and the capacity for high strain.

Others, such as U.S. Pat. No. 4,909,251 to Seelich, utilize a biologically compatible tenside addition to the fibrinogen composition, and optionally further proteins as well as adjuvants or additives, to reduce reconstitution times. The tenside is from the group of non-ionic, cationic, anionic or zwitterionic tensides and is present in an amount from 0.03 to 15% by mass based on the fibrinogen content. Additions such as this have been shown to be useful in preparations having a high content of fibronectin, a plasma protein which is difficult to dissolve.

However, there are still existing inherent disadvantages to the present system of hemostatic and tissue adhesive compounds. The therapeutic compositions of some fibrin sealants and agents still contain non-autologous, non-single donor human fibrinogen, that is they comprise fibrinogen derived, or pooled from multiple human donors. Because of the risk of viral disease such as AIDS, hepatitis B and C, these compositions are not in use in the United States. With various incidents of infection reported it is unlikely that these compounds would ever be released for use in the United States.

Accordingly, practitioners of the art have sought to provide autologous or single donor fibrinogen compositions to minimize the risk of viral infection. However, substantial variation in the fibrinogen content of such preparations has lead to difficulty in predicting, accurately, the clinically effective dose required.

An alternate resolution to the above mentioned risks characteristic of human plasma derived therapeutic products was to provide fibrinogen from a mammalian source other than humans. This, however, can result in a severe immune response. Even the currently available highly purified bovine fibrinogen compositions, such as those indicated in U.S. Pat. No. 5,330,974, contain some foreign antigen.

A significant improvement in the design of fibrin tissue adhesives would involve the complete elimination of such solution preparation and mixing time, eliminate the risk of viral transmission, and severe immunologic response. A tissue adhesive of this type would consist of a blood activated single agent that is usable in a dry form, utilizing genetically engineered fibrin monomer, thus avoiding the time and constraints of preparing and pre-mixing ingredients, as well as any risk of viral transmission. The present invention provides such an agent. The new agent is distinguished from previously described fibrin tissue adhesives in that it is prepared using a genetically engineered fibrin monomer rather than fibrinogen. This allows it to be lyophilized from a single solution containing all of its constituents, including thrombin. Consequently, the agent is available as a dry powder which is activated upon blood contact producing effective hemostasis and subsequent adhesion.

Numerous publications describe the successful use of a simplified fibrin glue consisting of fibrinogen, thrombin, calcium chloride and Factor XIII (Kjaergard, HK et al., Ann Thorac Surg, 55(2):543–44, 1993; Hartman, AR et al., Arch Surg, 127:357–59, 1992; Dahlstrom, KK et al., Plastic and Reconstructive Surgery, 89(5):969–976, 1992). If one assumes that sufficient Factor XIII will be present in the blood being lost at the site of injury, it is possible to prepare a fibrin glue for hemorrhage control consisting of fibrinogen, thrombin and calcium chloride. One problem in preparing a solution with these ingredients, however, is the formation of a fibrin gel directly from the interaction of fibrinogen and thrombin. Therefore, a solution containing fibrin monomer is used instead of its precursor fibrinogen.

In the early stages of polymerization, the fibrin monomer molecules attach to each other by loose hydrogen and hydrophobic bonds which can be broken apart with ease. It is at this point that the fibrin monomer is utilized. The loosely bound monomers are chemically disrupted and the mixture is sonicated or shaken until the gel is dissolved. The dissolved fibrin monomer is then used to prepare a solution with thrombin and calcium chloride. This solution is finally lyophilized to dryness to provide a single agent fibrin tissue adhesive.

This method of preparation is necessary because it is not feasible to simply mix separately lyophilized powders in the correct proportions. The principal difficulty is in uniformly mixing the ingredients. The amount of thrombin, for example, is quite small compared to the amount of clottable protein: thus, it would be extremely difficult to thoroughly blend the two powders to achieve a consistent mixture. The method of the inventors produces a uniform preparation by virtue of being lyophilized from a true solution of its constituents.

When the lyophilized agent is exposed to Factor XIII, present in the blood at the site of use, the fibrin monomer will precipitate as stabilized fibrin with the appropriate adhesive qualities. The added thrombin, of course, also induces platelet aggregation which assists in achieving hemostasis.

The formulation of the single agent tissue adhesive may have additions, such as aprotinin, an inhibitor of fibrinolysis, or antibiotics. Likewise, numerous "inert" additives (substances such as preservatives, dispersants or additional diluents) known in the art can be added to the therapeutic compositions of the invention, with the understanding that such substances are physiologically compatible.

The therapeutic compositions and methods defined by the present invention are useful in connection with any of the clinical applications where adhesives, sealants and hemostatic agents can be used. Tissue adhesion, sealing of tissue or hemostasis are induced in a mammalian patient at a site of treatment by contacting the treatment site with a therapeutically effective amount of composition. According to the practice of the invention, such effective amounts need not be equivalent to amounts that cause complete or permanent adhesion of tissue, causing total sealing of tissue boundary or complete arrest of bleeding or loss of tissue fluid from a tissue or tissue boundary. Rather, such compositions are within the scope of the invention if the use thereof provides at least a partial effect that is of benefit to the patient in the course of treatment.

Amount of agent necessary to perform clinical procedures

varies widely depending on, for example, the size of the treatment site, the nature of the condition in need of treatment and factors unique to each patient. It is accepted in the art that it is the skill of the clinical practitioners to determine for each patient and for each condition the amounts of the agent that are effective.

The lyophilized composition of the invention may be used directly in powder form by directly sprinkling the agent onto a wound site or surgical incision. As it reacts with the blood and tissue fluids at the site, it will effect a seal or hemostasis. This is typically useful when the site to be closed is small and blood loss is not rapid.

Additionally, the lyophilized composition may be applied to a wound or surgical incision by, for example, incorporation into a gauze pad, sponge, collagen or gel-type matrix or into a similar device in treating the area. This is useful in controlling bleeding due to deep tissue injury involving arterial blood loss. It may also be useful in the treatment of more superficial wounds in which external semi-occlusive dressings may be applied.

SUMMARY OF THE INVENTION

With the above in view, it is therefore among the primary objectives of this invention to provide a single agent fibrin based hemostatic and tissue adhesive agent comprised of recombinant fibrin monomer, Bovine thrombin, and Calcium Chloride, which is lyophilized to powder form, and is activated upon blood contact, at the site of tissue injury, producing effective hemostasis and subsequent tissue adhesion.

It is another object of the present invention to provide a hemostatic agent/tissue adhesive which utilizes a recombinant fibrin monomer thus eliminating the risk of pooled human plasma derived fibrin, as well as eliminating severe immunologic reactions to pooled mammalian derived fibrin.

It is still another object of the invention to provide a fibrin monomer hemostatic agent/tissue adhesive which can be applied directly to the site of injury, in powdered form, without reconstitution.

It is yet another object of the invention to provide a therapeutic single agent fibrin monomer tissue adhesive, tissue sealant, or hemostatic agent derived from the thrombin-fibrinogen polymerization reaction.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides for a lyophilized fibrin monomer containing therapeutic composition effective as a tissue adhesive, tissue sealant, or hemostatic agent. A therapeutic single agent fibrin monomer tissue adhesive is derived from the thrombin-fibrinogen polymerization reaction, which is then prevented from stabilizing.

Due to the structure of the coagulation cascade, the fibrin monomer may be obtained in vitro by a variety of approaches. First, it is known that fibrin monomer is formed when the protein enzyme, thrombin, acts on the protein fibrinogen. With its proteolytic capabilities, thrombin removes two low molecular weight peptides from each molecule of fibrinogen, forming a molecule of fibrin monomer. This monomer has the automatic capability of polymerizing with other fibrin monomer molecules, forming long fibrin threads within seconds. Unlike other tissue adhesive and hemostatic agents which rely on fibrinogen, the present invention makes use of the fibrin monomer, a totally separate molecule.

In the early stages of this polymerization, the fibrin monomer molecules attach to each other by loose hydrogen and hydrophobic bonds which can be broken apart with case. It is at this point that the fibrin monomer may be utilized. However, under normal conditions, the blood plasma of vertebrates contains the inactive precursor of an enzyme, factor XIII, which can stabilize or strengthen the fibrin gels by introducing covalent bonds between the monomers. Activated Factor XIII binds neighboring molecules by covalently joining the side chains of certain glutamine acceptors. As a result of this cross-linking, stabilized fibrin has somewhat different properties than fibrin monomers.

As the present invention employs fibrin monomer, as opposed to fibrinogen, which all other tissue adhesives and hemostatic agents employ, the problems inherent in a fibrinogen system are eliminated. The problems in using fibrinogen, in a lyophilized formula, include the formation of a fibrin gel in the solution prior to lyophyllization because of the presence of thrombin. Thus lyophilizing a solution containing fibrin monomer instead of its precursor, fibrinogen, provides a solution to the problem of a single agent tissue adhesive, hemostatic agent. This solution lyophilizes to yield a truly homogeneous powder. When exposed to blood, thrombin activation of Factor XIII will precipitate the dissolved fibrin monomer as insoluble fibrin. The added thrombin also induces platelet aggregation which assists in achieving hemostasis.

The way in which fibrin monomer is derived from the thrombin-fibrinogen polymerization reaction and prevented from stabilizing is done at a point in which the fibrin monomer is easily disrupted during the reaction.

Lyophilized fibrinogen and thrombin are combined in a water solution. The fibrinogen is first dissolved at room temperature in distilled water and then subjected to sonication. Thrombin was then reconstituted, per directions. After dissolution, thrombin solution is injected into the fibrinogen solution.

Polymerization ensues instantaneously to form a soft gel. At this point the fibrin monomers are loosely bound and can be disrupted easily. To accomplish this disruption, a 10% ammonium hydroxide solution is added to the above solution, and the mixture is then sonicated or agitated until the gel is dissolved. The resultant fibrin monomer solution is then ready to be mixed into a final solution containing both thrombin and calcium chloride. This allows the solution to be lyophilized from a single solution containing all of its constituents, including thrombin. The agent is thus composed of fibrin monomer, thrombin, and calcium chloride; while it is assumed that sufficient factor XIII is present in the blood being lost at the site of injury.

The present invention relies on the presence of Factor XIII in the patient's own blood. When exposed to Factor XIII, the lyophilized fibrin monomer combination will precipitate as insoluble fibrin. The present invention therefore provides for a blood activated system employed as a powder without the premixing of ingredients.

What is claimed is:

1. A single agent hemostatic tissue adhesive comprising a lyophilized powder of fibrin monomer from human recombinant fibrinogen, calcium chloride, and bovine thrombin.

2. The invention of claim **1** whereto said hemostatic tissue adhesive does not require preparation, additives, or time, prior to use.

3. The invention of claim **1** consisting essentially of a lyophilized powder of fibrin monomer from human recom-

binant fibrinogen, calcium chloride, and bovine thrombin.

4. The invention of claim 1 wherein said fibrin monomer is from a human recombinant fibrinogen source free of virus.

5. A method of eliminating the risk of immunologic reaction to fibrinogen comprising applying the composition of claim 4 to injured tissue.

6. A method of eliminating the risk of immunologic reaction to fibrinogen comprising applying the composition of claim 1 to injured tissue.

7. A method of using a hemostatic tissue adhesive comprising using the invention of claim 1 with gauze, sponges, or other wound dressings in combination.

* * * * *

Appendix D

Bactericidal Activity Testing of Chlorhexidine-Releasing Silicone Substrate

Microbiology Research Associates, Inc. 33 Nagog Park, Acton, MA 01720 • (508) 263-2624 • FAX (508) 263-2786

Determination of the Bactericidal Activity of Whalen Experimental Chlorhexidine Film Disc

Project #: Whalen 007

Client: Whalen Biomedical, Inc.
11 Miller Street
Somerville, MA 02143

Materials:
1. Letheen Agar (Difco)
2. Sterile forceps
3. Sterile empty bottle
4. Neutralizing Buffer (BBL)
5. Phosphate Buffer (AOAC)
6. Pipettor

Samples:
1. 1% Chlorhexidine Film
2. 2% Chlorhexidine Film
3. 3% Chlorhexidine Film
4. Negative Control Film

Test Organisms:
1. *Staphylococcus aureus (SA)* ATCC #6538
2. *Pseudomonas aeruginosa (PA)* ATCC #9027
3. *Streptococcus pyogenes (SP)* Clinical isolate

Method:

1. 0.5 ml of a 1:1000 dilution of test organisms in phosphate buffer was added to the bottom of a sterile empty petri dish and negative control dish.

2. Separately, each disc was place using sterile forceps directly on top of the 0.5 ml of test organism to create a "sandwich effect."

3. Each disc was allowed to have direct contact with the microorganisms for 1 min., 5 mins., 30 mins. and 1 hour at room temperature.

4. After the contact time interval, each disc was aseptically placed in a sterile empty tube. The remaining 0.5 ml of test organism was pipetted into the tube with the disc and the petri dish was washed with 9.5 ml of neutralizing buffer and added to the tube containing the disc. The contents of the tube were vortexed for 30 seconds and exposed to an ultrasound for 15 seconds.

5. Plate counts were performed on the time zero negative control, time 1, 5, 30 mins. and 1 hour negative controls and the experimental discs using serial dilution and the pour plate technique using letheen agar.

6. A percent bacterial reduction was determined for each disc by comparing the total count for the negative control disc to the experimental discs for each test organism.

Results: See Table I - III

Conclusion:

1. In general, it took a minimum of 60 minutes of contact time for the 2% and 3% chlorhexidine discs to show large (>99.90) percent bacterial reductions against all three test microorganisms compared to the negative control disc.

2. In general, the 3% chlorhexidine disc showed the largest percent bacterial reduction in the shortest contact times (5 min.) against *Pseudomonas aeruginosa*. The 3% chlorhexidine disc showed the largest percent bacterial reduction against *Staphylococcus aureus* and *Streptococcus pyogenes* in 30 minutes of contact time compared to the negative control.

3. In general, all the Chlorhexidine disc showed small percent reductions against the test microorganisms in 1 minute and 5 minutes of contact time (except 3% against *PA* at 5 minutes) compared to the negative control disc.

Conclusion continued:

4. In general, the 3% Chlorhexidine disc showed the greatest cidal activity against all test microorganism and cidal activity increased as contact time increased.

5. In general, cidal activity increased for each disc as contact time increased.

Comments/Observations/Recommendations

1. The next phase of testing should include determination for the cidal activity of the Chlorhexidine discs against test organisms in 5% serum, to mimic the effect of blood on the cidal activity of these disc.

2. Larger disc surface area would most likely increase the cidal activity of this experimental product.

10-15-96
Date

James J. Barbato, M.S., M.P.H.
President

Table I

Bactericidal Activity of Chlorhexidine Film Discs
vs. *Staphylococcus aureus* ATCC #6538

Sample #	Trial #	Contact Time	Total Count/ disc	% Red	Trial #	Total Count	% Red
Neg.Control	1	0	$4.2x10^6$	----	2	$4.1x10^6$	--
Neg.Control	1	1 min	$4.8x10^6$	----	2	$4.3x10^6$	--
1% CHG Disc	1	1 min	$5.2x10^6$	0	2	$4.7x10^6$	0
2% CHG Disc	1	1 min	$4.0x10^6$	16.7	2	$4.4x10^6$	0
3% CHG Disc	1	1 min	$1.81x10^6$	62.29	2	$3.4x10^6$	20.93
Neg.Control	1	5 min	$5.9x10^6$	----	2	$4.0x10^6$	--
1% CHG Disc	1	5 min	$3.3x10^6$	44.07	2	$3.8x10^6$	5.0
2% CHG Disc	1	5 min	$2.1x10^6$	64.41	2	$2.73x10^6$	31.75
3% CHG Disc	1	5 min	$6.5x10^5$	88.98	2	$2.75x10^6$	31.25
Neg.Control	1	30 min	$7.4x10^6$	----	2	$5.5x10^6$	--
1% CHG Disc	1	30 min	$6.8x10^5$	90.81	2	$1.3x10^6$	76.36
2% CHG Disc	1	30 min	$8.5x10^4$	98.85	2	$1.6x10^5$	97.09
3% CHG Disc	1	30 min	$3.7x10^3$	99.95	2	$3.0x10^3$	99.94
Neg. Control	1	1 hr	$5.6x10^6$	--	2	$5.6x10^6$	--
1% CHG Disc	1	1 hr	$7.8x10^3$	99.86	2	$2.68x10^4$	99.52
2% CHG Disc	1	1 hr	$2.55x10^3$	99.95	2	$1.03x10^4$	99.82
3% CHG Disc	1	1 hr	<10	>99.99	2	<10	>99.99

Table II

Bactericidal Activity of Chlorhexidine Film Discs vs. *Pseudomonas aeruginosa* ATCC #9027

Sample #	Trial #	Contact Time	Total Count/ disc	% Red	Trial #	Total Count	% Red
Neg.Control	1	0	$4.5x10^5$	----	2	$8.6x10^5$	--
Neg. Control	1	1 min	$2.21x10^5$	----	2	$9.3x10^5$	--
1% CHG Disc	1	1 min	$8.5x10^3$	96.15	2	$2.23x10^5$	76.02
2% CHG Disc	1	1 min	$3.3x10^2$	99.85	2	$1.28x10^4$	98.62
3% CHG Disc	1	1 min	$2.5x10^2$	99.89	2	$3.90x10^3$	99.58
Neg.Control	1	5 min	$5.3x10^5$	----	2	$1.61x10^6$	--
1% CHG Disc	1	5 min	$2.41x10^3$	99.54	2	$3.2x10^4$	98.01
2% CHG Disc	1	5 min	$2.6x10^2$	99.95	2	$5.7x10^2$	99.96
3% CHG Disc	1	5 min	$2.0x10^1$	>99.99	2	$9.0x10^1$	99.99
Neg.Control	1	30 min	$7.9x10^5$	----	2	$1.76x10^6$	--
1% CHG Disc	1	30 min	<10	>99.99	2	$1.13x10^4$	99.36
2% CHG Disc	1	30 min	$3.0x10^1$	>99.99	2	<10	>99.99
3% CHG Disc	1	30 min	<10	>99.99	2	<10	>99.99
Neg. Control	1	1 hr	$1.74x10^6$	--	2	$1.74x10^6$	--
1% CHG Disc	1	1 hr	$2.5x10^2$	99.99	2	$1.5x10^2$	99.99
2% CHG Disc	1	1 hr	<10	>99.99	2	<10	>99.99
3% CHG Disc	1	1 hr	<10	>99.99	2	<10	>99.9

Table III

Bactericidal Activity of Chlorhexidine Film Discs vs. *Streptococcus pyogenes* (Clinical isolate)

Sample #	Trial #	Contact Time	Total Count/ disc	% Red	Trial #	Total Count	% Red
Neg. Control	1	0	$4.9x10^6$	--	2	$4.9x10^6$	--
Neg. Control	1	1 min	$8.9x10^6$	--	2	$8.9x10^6$	--
1% CHG Disc	1	1 min	$6.4x10^6$	28.09	2	$4.0x10^6$	55.06
2% CHG Disc	1	1 min	$3.9x10^6$	56.18	2	$3.5x10^6$	60.67
3% CHG Disc	1	1 min	$3.0x10^6$	66.29	2	$3.0x10^6$	66.29
Neg.Control	1	5 min	$3.5x10^6$	--	2	$3.5x10^6$	--
1% CHG Disc	1	5 min	$1.92x10^6$	45.14	2	$2.01x10^6$	42.57
2% CHG Disc	1	5 min	$1.08x10^6$	69.14	2	$1.11x10^6$	31.71
3% CHG Disc	1	5 min	$6.5x10^5$	81.43	2	$6.4x10^5$	81.71
Neg.Control	1	30 min	$2.2x10^6$	--	2	$4.7x10^6$	--
1% CHG Disc	1	30 min	$5.2x10^5$	76.36	2	$2.48x10^5$	94.72
2% CHG Disc	1	30 min	$5.5x10^4$	97.50	2	$8.5x10^4$	98.19
3% CHG Disc	1	30 min	$1.76x10^3$	99.97	2	$3.5x10^2$	99.99
Neg. Control	1	1 hr	$6.2x10^6$	--	2	$6.2x10^6$	--
1% CHG Disc	1	1 hr	$2.56x10^5$	95.87	2	$2.78x10^5$	95.52
2% CHG Disc	1	1 hr	$1.6x10^2$	>99.99	2	$1.5x10^2$	>99.99
3% CHG Disc	1	1 hr	$3.0x10^1$	>99.99	2	$4.0x10^1$	>99.99

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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PHYLIS M. RINEHART
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